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With 16 Illustrations

LONDON

J. & A. CHURCHILL Ltd.

104 GLOUCESTER PLACE, W. 1

Reprinted 1947

Published April, 1940
Second Printing 1950

SET UP AND ELECTROTYPED BY T. MOREY & SON
PRINTED IN THE UNITED STATES OF AMERICA

PREFACE

The term inflammation [illegible] interdependent reactions [illegible] by the [illegible] vertebrate tissue of a foreign body [illegible] The injurious agent may be [illegible] exogenous or even of end[illegible] such, is a basic phenomenon in [illegible] analysis of the mechanisms involved [illegible] to the organism as a whole, [illegible] biological concepts, is [illegible] Inflammation is essentially [illegible] manifestations of cellular injury. It [illegible] response on the part of normal [illegible] damage. There are [illegible] injury. Neoplasia, for instance, may [illegible] resultant of such a form. C[illegible] merely another form of [illegible] injurious stimuli. As we [illegible] further [illegible] mechanisms of cellular physiology [illegible] ceivable that the term inflammation may [illegible] and may therefore eventually be [illegible] distinct types of cellular injury may [illegible] as quantitative variations of a single [illegible] basic physiological disturbance. What [illegible] an inflammatory reaction may in the [illegible] only as a prototype of cellular injury [illegible] animals. Reparative reactions may [illegible] and considered from a similar angle. H[illegible] stage of development the writer believes that [illegible] flammation may still be retained [illegible] designation for a series of fundamental [illegible] which tend to localize and finally dispose of [illegible] agent.

primarily for use with capillary blood, but they are of course applicable to samples of venous blood.

Among the methods of blood analysis will be found certain procedures, i.e., cholesterol, calcium and CO_2-combining power, which have been adopted, unaltered, from the published descriptions of the original authors. Although we have introduced no modification or improvement in these methods, we have felt it advisable to include them for the sake of completeness, and in order to have together a set of instructions which make it possible to execute all the determinations which are commonly asked for in routine laboratory investigations. For the same reason, the tests and estimations on CSF and faeces are included, and those urine examinations which are essential for the simpler physiological tests of function.

The functional tests given are described in sufficient detail to enable them to be executed with precision, but no great space is devoted to discussing their clinical significance. Likewise no attempt is made to give a complete statement of the amounts of the various substances present in blood in diseased conditions nor to describe their significance at any length. Brief mention is made only of those clinical conditions in which abnormal values are most commonly encountered.

My former colleagues, Dr. G. A. D. Haslewood, Mr. G. E. Delory and Dr. D. Beall, have had an intimate share in the development of the procedures presented in this book. On their ideas and efforts I have largely depended for the elaboration of new analytical principles and the modification of existing ones to the needs of the micro-techniques. To them and to other colleagues, assistants and students, my thanks are due, for their counsel, collaboration, and loyal support.

E. J. K.

Reinforced by the modern methods of experimental biology, particularly as developed in the dynamic sciences of physiology, biochemistry, and immunology, classical pathology is now faced with the task of unravelling the various basic problems of inflammation, neoplasia, degenerative, and metabolic diseases. This monograph is offered as an attempt to analyze and correlate earlier and recent observations pertaining to the development of the inflammatory reaction. This endeavor at bringing unity and therefore at formulating a rational concept of inflammation doubtless meets with considerable shortcomings and omissions. The writer, therefore, feels compelled of necessity to rely on the indulgence of his readers for what may appear to have been oversights. This is to be attributed in part to the wide scope of the field under investigation, and to the abundance of a ramifying literature which has rendered it indispensable to omit some of the indirectly related material. To be certain, some of these studies are not only interesting, but also represent valuable contributions. At the same time, their subject matter extends somewhat beyond the central theme of this monograph. For the sake of clarity and emphasis their omissions became, in many instances, a necessity.

The writer wishes to take this opportunity of expressing his acknowledgment for considerable aid received throughout the course of the work, the results of which are contained in this monograph. I am particularly indebted to Professor S. Burt Wolbach who kindly read some of the chapters of the original manuscript and who for the past ten years has placed at my disposal in the Department of Pathology many of the necessary facilities for properly conducting the experimental work. It is with considerable pleasure that I recall the two years spent at the Henry Phipps Institute of the University of Pennsylvania with Professor E. L. Opie. It is under his stimulating influence that I began my experimental investigation on inflammation. I am likewise considerably indebted to Professor Walter B. Cannon for kindly suggesting to the Editorial Board of the Experimental Bi-

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CHAPTER I

NORMAL VALUES

By "normal value" is meant the amount of a constituent present in the body fluid or excretion of a healthy human being. In fact, this amount varies over a range, and while most healthy persons can be included in a class having the accepted "normal" amount, some individuals are found to show divergent figures. Such exceptional individuals are often entirely "normal" in all other investigated respects. The judgment, therefore, of whether a given analytical figure is "normal" will depend on the experience and total data at the command of the interpreter of the result.

The values given below are taken from the literature and from our accumulated results. In most cases they are assumed to apply to the method of analysis given here. This assumption is generally the result of a direct comparison between the method given and a method which has been "standardized for normal human beings"; in other cases, inspection of a large collection of determinations prompts the feeling that the findings of a modified method do not differ significantly from those "accepted." It must be emphasized, however, that in several cases (uric acid in blood, for example,) no reliable data of the range of "normal value" exist; it is hoped that when more general agreement as to methods to be used has been attained, such data will be forthcoming. Blood values are for fasting persons.

TABLE 2.—ABNORMALITIES IN COMPOSITION OF HUMAN BLOOD

Constituent	Clinical conditions in which high values (unless otherwise stated) are found
Plasma proteins (total)	Anhydremia. Low in nephritis with oedema (nephrosis), starvation.
Plasma albumin	Low in nephrosis.
Plasma globulin	Nephrosis, anaphylactic conditions.
CO_2-combining power	Alkalosis ($NaHCO_3$ administration, intestinal obstruction, over-breathing). Low in acidosis (diabetes, starvation, and severe nephritis).
Sugar	[illegible]
Non-protein N	[illegible]
Urea	[illegible]
Uric Acid	[illegible]
Creatinine	[illegible]
Chlorides (whole blood)	[illegible]
Chlorides (plasma)	Low in pneumonia, fever, diabetes; all cases of dehydration, such as gastro-intestinal disturbances associated with diarrhoea and vomiting.
Phosphates as P	Nephritis. Low in rickets.
Calcium (serum)	Hyperparathyroidism. Low in tetany (infantile), parathyroidectomy, severe nephritis, coeliac disease.
Cholesterol	Biliary obstruction, nephritis, nephrosis, diabetes, pregnancy. Low in pernicious anaemia.
Phosphatase	Generalized bone disease, obstructive jaundice.
Bilirubin	Jaundice.

UREA

Urea represents about 50 per cent. of the non-protein nitrogen of the blood. Normally there are between 20 and 40 mg. of urea present per 100 ml. High values are found in conditions associated with impaired renal function—particularly in chronic nephritis, but also in some cases of acute nephritis, prostatic obstruction, cardiac failure, etc.

PRINCIPLE

The sample of blood is digested with urease, and the urea thus converted into ammonia. After the removal of proteins, the colour produced by the ammonia with Nessler's reagent is compared colorimetrically with the colour produced under the same conditions with a standard ammonium chloride solution.

Direct Nesslerization does not lead to the production of cloudiness in the case of protein-free filtrates from unlaked blood. This is due to the fact that the sulphydryl substances, glutathione and ergothioniene, which produce turbidities with Nessler's reagent because of the insolubility of their mercury salts, are confined to the cells and do not appear in the filtrate, as is the case with filtrates of laked blood. Filtrates of unlaked blood have the further advantage that no ammonia is contributed to the determination through the action of the arginase of the red cells on the arginine contained in most commercial preparations of urease (see Addis, 1928). The use of zinc hydroxide as deproteinizing reagent eliminates a small amount of turbidity-producing substance contributed by most preparations of urease.

METHOD

0·2 ml. of blood is added to a centrifuge tube containing 3·2 ml. of isotonic sodium sulphate solution.

A "knife-point" (about 20 mg.) of Jack Bean meal is added, and the tube stoppered with a rubber bung, mixed, and incubated at 37° C. for 20 minutes. 0·3 ml. of zinc

SOLUTIONS

Nessler's Reagent.—11·25 g. of iodine crystals are dissolved in a solution of 15 g. of potassium iodide in 10 ml. of water. 15 g. of mercury are added, and the mixture, kept cool in water, is shaken until the supernatant liquid has lost its yellow colour. This supernatant liquid is then decanted into a 100 ml. flask and a drop tested with 1 per cent. starch. If no colour is obtained, more iodine solution (prepared as above) is added until a drop of the mixture gives a faint reaction with starch.

The total solution is then diluted to 100 ml. and poured into 485 ml. of 10 per cent. sodium hydroxide. If the solution is turbid, it should be allowed to settle before use.

Standard Ammonium Chloride Solution (containing 0·01 mg. of nitrogen per ml.).—153 mg. of pure ammonium chloride are weighed out and dissolved in water. The volume is made to 100 ml.; 25 ml. of this solution with 10 ml. of N-sulphuric acid are diluted to 1 litre with distilled water.

Isotonic Sodium Sulphate.—Thirty g. of crystalline sodium sulphate ($Na_2SO_4.10H_2O$) are dissolved in water and made to 1 litre.

Zinc Sulphate.—Ten g. of crystalline zinc sulphate ($ZnSO_4.7H_2O$) are dissolved in water and made to 100 ml.

0·5 N-*Sodium Hydroxide.*—This should be accurately prepared, and should be titrated against the zinc sulphate. 10·8–11·2 ml. should be necessary to produce a permanent pink colour with phenolphthalein, when titrated into 10 ml. of zinc sulphate diluted with water.

NON-PROTEIN NITROGEN

The non-protein nitrogen containing substances of blood are urea (10–20 mg. N), uric acid (1–2 mg. N), creatinine (0·5–1 mg. N), amino-acid nitrogen (6–8 mg.), and substances such as glutathione and ergothionienc (5–10 mg. N per 100 ml. of blood). The normal range of non-protein (N.P.N.) is from 25–40 mg. per 100 ml. Increased values are found in the conditions showing a high blood urea.

in the development of the inflammatory reaction will be discussed, particular attention being paid to the orderly sequences that occur from the initial introduction of an irritant until its ultimate disposal. The importance of inflammation as a significant factor regulating bacterial invasiveness will likewise be considered.

Historical survey of the problem. The subject of inflammation has always played a dominant part in the history of medicine. *Hippocrates* (about 460–377 B.C.) regarded inflammation as being closely associated with fever. There is no uniform term in the Corpus Hippocraticum by which to designate inflammation. For the local heat the terms *phlegmone*, *phlogosis* or *phlegmasia* were employed. The swelling was called *oidema* or *onkos;* and the redness was termed *erysipelas*. The inflammatory process was included in the Hippocratic doctrine of Apostasis or the so-called "deposit" theory. A general disease was classified either as a fever or as an epidemic malady. According to this view the irritating material was rendered harmless by being usually deposited in some local cutaneous area thus forming a suppurative abscess. The noxious substance was believed to be locally eliminated by the process of "coction." Suppuration promoted the termination of the general malady by a special digestive mechanism (coction). Thus the idea of parenteral digestion in inflammation, which has been particularly stressed in modern times, dates as far back as the Hippocratic era. The Corpus Hippocraticum erroneously regarded inflammation as the *result* of the generalized febrile state rather than the cause. Both fever and inflammation were considered as healing processes which, however, might prove harmful when enhanced beyond certain limits. According to this theory the nature of any malady depended on a disturbance involving one or more of four humors (blood, phlegm, yellow and black bile). On the basis of this doctrine, inflammation was interpreted as an excess or as a distortion of one of these humors. This viewpoint was essentially accepted by Galen several centuries later; and

(2) "High" standard:

$$\text{N.P.N.}^{*}\begin{cases} = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 0{\cdot}05 \times \dfrac{100}{0{\cdot}05} \\ = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 100 \end{cases}$$

* mg. per 100 ml. of blood or plasma.

SOLUTIONS

Nessler's reagent and *ammonium chloride* standard as described for urea.

Fifty per cent. Sulphuric Acid containing Selenium Oxide.—Fifty ml. concentrated acid are allowed to run slowly and with shaking into 50 ml. of distilled water in a 100 ml. volumetric flask. Selenium oxide (1 g. SeO_2) is added and shaken until dissolved. The mixture is cooled to room temperature, made to the mark, and mixed.

URIC ACID

Uric acid is normally present to the extent of 2–4 mg. per 100 ml. of blood. In gout and in certain conditions of renal impairment high values are found.

PRINCIPLE

Blood in isotonic sodium sulphate solution is treated with a phosphotungstic acid reagent. This precipitates the proteins, and on addition of sodium cyanide to the supernatant liquid, the excess of phosphotungstic acid reagent produces a blue colour with the uric acid present. The colour is compared with that given by a standard solution of uric acid. The blood must not be laked, as interfering substances, such as glutathione and ergothionienc, would be liberated from the cells.

METHOD

0·2 ml. of capillary blood is pipetted into 8·2 ml. of isotonic sodium sulphate in a 15 ml. centrifuge tube. 0·6 ml. of Folin's (1934) uric acid reagent is added. The tube is

portant principles to Celsus' cardinal points, namely the *etiologic* and the *teleologic* significance of inflammation. Hunter believed that inflammation could be caused by *any* injury. The deleterious agent might be pressure, friction, heat, cold, corrosion, or air in wounds. (He regarded inflammation as an adaptive defensive mechanism which ultimately restored the injured part to its normal function.) "But if inflammation develops, regardless of the cause, still it is almost the same type everywhere, because everywhere it is an effect whose purpose it is to restore the parts to their natural functions." Hunter seems to have been the first to realize the significance of "coagulable lymph" in limiting the extent of injury. He pointed out that if the coagulable lymph is secreted from the blood an *adhesive inflammation* results. Experimental evidence will be brought forward in subsequent chapters throwing further light on this early view concerning *the localizing tendency of an inflammatory process.*

In the latter part of the eighteenth and the beginning of the nineteenth century the teleologic concept of Hunter was *relegated to the background. Particularly under the influence* of the French, notably Broussais (1772–1838) and Gendrin (1796–1890), the inflammatory reaction was *regarded as the disease itself which consequently* had to be attacked.

Even the great pioneers of the nineteenth century, Samuel, Cohnheim, and Virchow, emphasized the physiological changes in the local circulation of an inflamed area or the alteration in the appearance of the cell without particularly stressing the defensive mechanisms involved in these local changes. Samuel, for instance (1833–1899), stated his belief as follows: "The alteration of the vessel walls in all the changes of phenomena is recognized as the innermost nucleus to which the most important and characteristic events, the congestion and exudation, owe their origin." Cohnheim (1839–1884) stands out as one of the most penetrating students of the subject of inflammation. *His contributions* display a profound understanding of some of the principal

Sodium hydroxide solution (40 g. per 100 ml.) is now added to the hot solution until the latter is alkaline to phenolphthalein. The cooled solution, filtered if necessary, is treated with 200 ml. of absolute alcohol, and allowed to stand for 24 hours. The white crystals are filtered off and dried in a desiccator.

2. Preparation of reagent: 100 g. of molybdate-free sodium tungstate are treated gradually with a solution of 30 ml. of "syrupy" phosphoric acid (89 per cent.) in 150 ml. of water. The mixture is boiled gently under reflux for 1 hour, decolourized as above with a drop of bromine, cooled and diluted to 500 ml.

Stock Uric Acid Standard (Folin) (= 1 mg. per ml.).—One g. of uric acid is placed in a 1-litre flask. 0·6 g. of lithium carbonate is dissolved in 150 ml. of cold water. The carbonate solution, filtered if necessary, and warmed to 60° C., is added to the flask containing the uric acid, which is warmed under the hot tap. The warm mixture is shaken for five minutes, cooled at once under the tap, and treated with 20 ml. of formalin (40 per cent. solution of formaldehyde) and enough water to fill half the flask. A few drops of methyl orange are added, and then, gradually with shaking, 25 ml. of N-sulphuric acid. The solution should turn pink when 2–3 ml. of acid remain to be added. The mixture is now diluted to 1 litre, mixed and stored in the dark in a stoppered bottle, when it will keep almost indefinitely.

Uric Acid "Blood" Standard (= 0·004 mg. per ml.).—Two ml. of the above "stock" standard solution are diluted with water and 1 ml. of 40 per cent. formalin to 500 ml. This solution should be made up fortnightly.

CREATININE

Colorimetric Estimation

PRINCIPLE

Creatinine gives a red colour with alkaline solutions of picric acid (Jaffe's reaction). A similar colour is also given by blood (and plasma) filtrates. It is not certain that the

Virchow's views on inflammation have been abandoned and are now merely considered of historical interest. If they are briefly restated here it is due not so much to their significance but rather to the general influence which Virchow exerted on the scientific thought of his time. To Virchow (1821–1902) the important change in injury consisted in the swelling of the parenchymatous structures. The inflammatory irritant was regarded as a nutritive factor whereby the cells become capable of attracting various substances from the circulating blood. The cell soon displays "cloudy swelling." The inflammatory swelling is largely referable to the increase in cellular volume. Virchow failed to see the importance of the disturbance in the filtration of the small vessels or the protective and restorative function of inflammation. According to him the inflammatory reaction was largely a degenerative process: "Above all I also assert the *degenerative character* of inflammation and, although I designate it as an increased nutritive act, I do not see in it any sign of increased power, but much more an expression of its decrease, the basis of diminution and not rarely complete destruction of the function of parts."

A different theory to explain inflammatory processes was advanced in the latter part of the nineteenth century by E. Metchnikoff. This investigator was primarily a zoölogist, and his views represented the outcome of careful observations in the realm of comparative pathology. The biological interpretation of inflammation as an adaptive reaction resulting from natural selection was the stand adopted by Metchnikoff. He rejected the "nutritive" theory of Virchow and the views of Samuel and Cohnheim that the inflammatory reaction was essentially referable to alteration in the permeability of the vascular wall. To Metchnikoff the primary function of inflammation involves the phagocytic capacity of leukocytes which dispose of the bacterial irritant. The struggle between white cells and the irritating agent in the affected tissue constitutes the *primum movens* of the inflammatory reaction. The enhanced permeation through the

CREATININE

Photometric Estimation

The amount of creatinine in blood is so small that the amount of colour in a test, which is contributed by the excess of reagent (alkaline picrate solution), is appreciable compared with that produced by the reaction of creatinine with reagent. The difficulty of obtaining an accurate assessment of the colour due to creatinine can be overcome by using the colorimeter as a photometer in the manner described by Delory and Jacklin (1942).

The neutral grey screen (density 0·50; cf., p. 142) is placed on the left-hand rack of the colorimeter, and the Ilford blue-green filter placed over the eyepiece. The standard solution is placed in the right-hand cup and its depth is adjusted until the two fields appear equal. The reading S is recorded. The test solution is now substituted in the cup to give the reading T. Finally, a blank consisting of 1 ml. of water and 0·5 ml. of alkaline picrate solution is read (reading B).

CALCULATION

$$\text{Blood ``creatinine''}^{*} \begin{cases} = \dfrac{1/T - 1/B}{1/S - 1/B} \times 0{\cdot}001 \times \dfrac{100}{0{\cdot}1} \\[2ex] = \dfrac{\dfrac{B - T}{BT}}{\dfrac{B - S}{BS}} \times 1 \\[2ex] = \dfrac{S}{T} \times \dfrac{B - T}{B - S} \times 1 \end{cases}$$

* mg. per 100 ml. of blood.

If it is desired to avoid this calculation, a curve is made from the readings obtained with varying strengths of standard. Results can then be read off from this curve. Since different preparations of picric acid may contain varying amounts of impurities (e.g., dinitrophenol), it is advisable to check the curve for each new batch of picric acid.

CHAPTER II

THE MECHANISM OF CAPILLARY FILTRATION

As pointed out in the preceding chapter, an acute inflammatory reaction is the resultant of a series of events induced in tissues by the presence of an irritant. These steps occur in an orderly sequence leading ultimately to the localization and disposal of the irritant. Inflammation is characterized by an initial disturbance in local fluid exchange. This is primarily referable to an alteration in the normal filtration equilibrium between the small vascular channels, the lymphatics, and the so-called intercellular fluid. Before considering the changes which an inflammatory irritant induces on the capillary vessels a brief survey of the mechanism of physiological filtration will be presented.[1]

The fundamental work of Krogh (1922, 1929) dispelled the earlier view that capillaries were merely inert thin-walled tubes whose blood supply largely depended on arteries and arterioles. This investigator demonstrated the independent contractility of capillaries and their capacity to respond individually to the demands of adjacent tissues. This property is doubtless of considerable significance in grading filtration of fluids through a given capillary wall.

The most satisfactory hypothesis that explains the filtration equilibrium between blood and tissue fluid was offered by Starling (1896). This investigator called attention to the fact that crystalloidal substances, such as urea, glucose, salts, or creatinine, readily pass through the capillary wall and are found in approximately equal concentrations in the blood and intercellular fluid. The plasma proteins, however, owing to their greater molecular dimensions are relatively

[1] For an excellent detailed consideration of normal capillary filtration the reader is referred to the recent reviews by E. M. Landis (1934, 1937).

molybdate is reduced to give a blue substance. The amount of blue colour produced in the solution is directly proportional to the amount of phosphate present.

METHOD

0·2 ml. of whole blood or plasma is pipetted into 3·2 ml. of water or isotonic sodium sulphate and treated with 0·6 ml. of 25 per cent. trichloroacetic acid. The mixture is shaken well, and after 5 minutes filtered through a small paper. 2 ml. of the clear filtrate (= 0·1 ml. of blood or plasma) are treated at the same time as 2 ml. of the dilute standard phosphate solution (= 0·004 mg. P) with 0·3 ml. of the ammonium molybdate solution followed by 0·2 ml. of the reducing agent (aminonaphtholsulphonic acid). The contents of the tubes are gently shaken between each addition and the colours are read in a colorimeter after 10 minutes (red or orange light filter).

CALCULATION

$$\text{Blood phosphate}^{*} \begin{cases} = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 0{\cdot}004 \times \dfrac{100}{0{\cdot}1} \\ = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 4 \end{cases}$$

* mg. per 100 ml. of blood.

SOLUTIONS

Trichloroacetic Acid Solution.—Twenty-five g. of pure trichloroacetic acid dissolved in water, and diluted to 100 ml. and mixed.

Acid Ammonium Molybdate.—Five g. ammonium molybdate are added to a mixture of 75 ml. distilled water and 15 ml. of concentrated sulphuric acid in a 100 ml. volumetric flask. The mixture is shaken until dissolution is complete and cooled to room temperature. The solution is then made up to 100 ml. and mixed.

Reducing Agent. 0·2 per cent. aminonaphtholsulphonic

The blood pressure in the arterioles is distinctly higher than that in the venules. This indicates that there is a fall in pressure along the course of the capillary. It is therefore quite conceivable that the establishment of a capillary gradient may favor filtration of fluid at the arteriole end of the capillary and reabsorption at the venous end. The latter would be expected from the Starling hypothesis only if the osmotic pressure of plasma proteins exceeds the level of capillary pressure at the venous end. This relationship was particularly stressed by Landis (1926) and by Schade (1924).

Direct measurements by Landis have largely verified the Starling view. By introducing a micro-pipette directly into the lumen of a capillary in the exposed mesentery of a frog this investigator was able to show that the pressure on the arteriolar end of the vessel averages 14.5 cm. H_2O. On the venous end the level was somewhat lower, averaging 10.0 cm. H_2O. White (1924) estimated the osmotic pressure of the plasma proteins for frog's blood to be between 10 and 12 cm. of water. Landis (1927) reported an average figure of 11.5 cm. On the basis of these measurements Landis concluded that Starling's theory could readily explain the movement of fluid through the capillary wall. The gradient of pressure along the course of the minute vessels favors filtration at the arteriolar portions of the capillary and reabsorption at its distal part. The rate of fluid passage appears to depend on the balance between the capillary pressure and the osmotic pressure of the plasma proteins. These original conclusions were based on an assumption that the endothelial lining was similar to an inert membrane and that it was essentially impermeable to the outward passage of proteins.

Churchill, Nakazawa, and Drinker (1927) demonstrated that the skin capillaries of the frog at any rate were not wholly impermeable to the leakage of proteins. These investigators recovered from the lymph appreciable amounts of protein which markedly reduced the effective osmotic pressure of the plasma colloids. They found an average

METHOD

0·2 ml. of blood is pipetted drop by drop into a centrifuge tube containing a mixture of 8 ml. of absolute alcohol and 2 ml. of ether. The stoppered tube is shaken vigorously for about 1 minute, and is then allowed to lie horizontally, with an even distribution of the precipitate along the tube, for 30 minutes. The mixture is then centrifuged, and the supernatant liquid poured, as completely as possible, intc a small beaker. This is placed on a water-bath or hot plate, and the contents carefully evaporated to dryness. The residue is washed out with about 4 ml. of chloroform, in three portions, into a glass-stoppered 10 ml. measuring cylinder, and the volume made to 5 ml. with chloroform.

0·5 ml. (≡ 0·5 mg. of cholesterol) of the cholesterol standard is added to a similar container and diluted to 5 ml. with chloroform. To each solution are added 2 ml. of acetic anhydride, and 0·1 ml. of concentrated sulphuric acid. The cylinders are stoppered, and their contents mixed and allowed to stand in the dark for 10 minutes. The two solutions are then compared in the colorimeter. (Red or orange light filter.)

CALCULATION

$$\text{Total cholesterol}^{*} \begin{cases} = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 0\cdot5 \times \dfrac{100}{0\cdot2} \\ = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 250 \end{cases}$$

* mg. per 100 ml. of blood.

Cholesterol Standard.—0·1 g. of pure cholesterol in 100 ml. of chloroform.

GLUCOSE

Titrimetric method for "True Sugar"

Harding's (1932, 1933) modification of the Schaffer-Hartmann method. This method gives "true sugar values" as opposed to total reducing substances when applied to the filtrate of unlaked blood described below.

gators find it difficult to agree with Drinker and Field (1931) that lymph and tissue fluid are essentially identical. They believe that lymph has usually a higher protein concentration than the tissue fluid bathing the outside of the capillary wall. They ascribe wide variations to the protein concentration of lymph. These variations may be referable to such factors as the rate of filtration and the relative amount of absorption. The slower the filtration and the more complete the reabsorption, the more will the lymph differ in composition from the tissue fluid. The converse state of affairs would favor a closer identity to the composition of the two fluids. Drinker and Field (1933) in answer to these criticisms bring forward evidences indicating that the protein concentration of lymph is subject to wide fluctuations. For instance in active motion the concentration becomes appreciably reduced owing to dilution of the capillary filtrate. They reiterate their view that the tissue fluid may likewise show variations ranging roughly between 0.3 and 4.0 per cent of protein. It is their belief that since lymph is of similar composition it follows the same fluctuations in protein content, depending on factors such as changes from rest to activity, or alteration in the permeability of the blood capillaries. The difficulty in the solution of this question seems to reside primarily in the inability to obtain normal tissue fluid for protein determination.

The inferences on the relative impermeability of the human capillary wall to proteins are drawn from observations on the changes in filtration rate produced by fluctuations in venous pressure and in the colloid osmotic pressure of the blood The correctness of this view would indicate that the osmotic pressure of the plasma proteins in the human is the primary effective force in inducing absorption of fluid. The measurements would thus completely support the Starling theory. It is also interesting to note that to cause a dominance of filtration a rise of 25 cm. H_2O (or 18 mm. of mercury) in the venous pressure is fully sufficient. This indicates that a normal human is always fairly close to the

($\frac{3}{4}$ inch) test-tube. A "blank" is prepared with 2 ml. of distilled water and 2 ml. of reagent. Both tubes, stoppered lightly with cotton wool, are placed in a boiling water bath for exactly 10 minutes. They are then cooled at once under the tap. To each is added 1 ml. of 2 per cent. potassium iodide and 1 ml. of 2 N-sulphuric acid. After standing 1 minute the contents of each tube are titrated with N/200 sodium thiosulphate. One per cent. soluble starch (made up in water or, better, in saturated phenol red solution) is used as indicator. The titration figure of the test solution is subtracted from that of the "blank."

CALCULATION

1 ml. N/200 thiosulphate = 0·116 mg. glucose

The ml. of thiosulphate given by the difference between the "blank" and "test" titrations is equivalent to the amount of glucose present in the "test." Hence :—

ml. N/200 thiosulphate × 0·116 = mg. glucose in 2 ml. filtrate (i.e., in 0·1 ml. blood)

And therefore :—

$$\left.\begin{array}{l}\text{ml. N/200 thiosulphate}\\ \quad \times\ 0{\cdot}116 \times \dfrac{100}{0{\cdot}1}\end{array}\right\} = \text{mg. of glucose per 100 ml. blood}$$

i.e.,

$$\left.\begin{array}{l}\text{ml. N/200 thiosulphate}\\ \quad \times\ 116\end{array}\right\} = \text{mg. of glucose per 100 ml. blood.}$$

If the blood-sugar value thus obtained is greater than 400 mg./100 ml., the determination should be repeated, using as test solution a mixture of 1 ml. of filtrate and 1 ml. of water. The result then obtained is multiplied by 2.

SOLUTIONS

Isotonic Sodium Sulphate-Copper Sulphate Solution (to prevent glucolysis).—A mixture of 320 ml. of 3 per cent. sodium sulphate and 80 ml. of 7 per cent. copper sulphate ($CuSO_4.5H_2O$).

Hesselmann (1932) was able to confirm the presence of the gradient in the tongue and mesentery of urethanized frogs. Contrary to the findings of Rous who reported that nerve section fails to obliterate the gradient, Hesselmann claimed that upon cutting the sympathetic nerve to the tongue, the gradient disappears. The possibility, however, of injury to capillaries by both urethane and prolonged exposure is not precluded in the experiment of Hesselmann.

On the basis of his results, Rous cast serious doubt on the validity of the Starling hypothesis as an adequate explanation of fluid exchanges. Rous and Smith (1931) failed to confirm Landis' observation on the passage of trypan red (vital red HR) through the capillary wall of the frog mesentery. Landis (1927*a*) reported that this dye frequently escaped from an individual capillary "in greater amount outside the arterial portion of the vessel." In view of the elevated pressure at the arterial end of the capillary, this was interpreted as additional evidence supporting the pressure gradient hypothesis. Rous and Smith correctly pointed out the possibility of capillary injury and distortion as a result of prolonged exposure to physiological solution. The findings of Rous and Smith in this connection were consistent. Trypan red, as well as trypan blue and Chicago blue 6B, escaped first from the distal portion of the capillaries and the smallest venules. Landis has recently criticized these observations by remarking on the large amounts of dye utilized by Rous and Smith and on their failure to measure capillary pressure (1934). It is difficult to see how the amount of dye injected would be the conditioning factor in inducing the formation of the characteristic permeability gradient. Landis (1934) insists that observations made with relatively poorly diffusible dyes should not be stressed too far in the evaluation of their results: "The gradient is therefore demonstrable only with dyes whose diffusibility is much less than that of the blood constituents concerned in nutrition. To what extent observations with poorly diffusible dyes can justifiably be applied to the highly diffusible

results obtained are identical with those found with the previously described titration method. The proteins are precipitated by sodium tungstate and copper sulphate (Somogyi, 1931) and the filtrate is treated with a modified Harding and Downs (1933) copper reagent, from which the iodate is omitted. The cuprous oxide formed is estimated by the colour produced with an arseno-molybdic acid solution (Nelson, 1944).

METHOD

0·05 ml. of whole blood is pipetted into 1·85 ml. of isotonic sodium sulphate-copper sulphate solution in a conical centrifuge tube. 0·1 ml. of sodium tungstate is added, and the mixture is well shaken. The precipitated proteins and copper tungstate are spun down in the centrifuge. 1 ml. of the supernatant fluid (≡ 0·025 ml. of blood) is mixed with 1 ml. of the mixed copper reagent in a ¾ in. diameter test-tube. The tube, stoppered with cotton wool, is placed in a boiling water-bath for exactly 10 minutes. After immediate cooling, 3 ml. of the arseno-molybdic acid reagent are added. The colour is compared, after 10 minutes, with that produced by 1 ml. of a standard glucose solution in benzoic acid, treated in the same way as the blood filtrate. (Red or orange filter.)

CALCULATION

Let X = concentration of standard in mg. per ml.

$$\text{Blood sugar}^{*} = \frac{\text{Reading of standard}}{\text{Reading of test}} \times X \times \frac{100}{0{\cdot}025}$$

e.g., with 0·02 mg. per ml. standard, Blood sugar *

$$\left\{\begin{aligned} &= \frac{\text{Reading of standard}}{\text{Reading of test}} \times 0{\cdot}02 \times \frac{100}{0{\cdot}025} \\ &= \frac{\text{Reading of standard}}{\text{Reading of test}} \times 80 \end{aligned}\right.$$

Similarly, with 0·05 mg. per ml. standard, Blood sugar *

$$= \frac{\text{Reading of standard}}{\text{Reading of test}} \times 200$$

* mg. per 100 ml. blood.

CHAPTER III

THE CAPILLARIES IN INFLAMMATION

The introduction of a chemical or bacterial irritant into normal tissue is followed by definite disturbance in the filtration equilibrium. There is a local increase in the passage of plasma as evidenced by the edema which may develop fairly early. Both the capillary pressure and the permeability gradient are so profoundly altered as to favor enhanced fluid accumulation in the affected tissue. The principal forces at play in promoting these changes may be listed as follows: (1) Increased capillary pressure accompanying local vasodilatation; (2) Increase in the actual number of functioning capillary channels; (3) Increase in capillary permeability. The frequently encountered initial constriction of the small vessels will not be discussed here owing to the lack of exact data on the mechanism and significance of this phenomenon. The rôle of lymphatics in the early and later stages of the inflammatory reaction is of such importance for a clear understanding of fluid exchange in injury as to warrant a separate and detailed description in a subsequent chapter. For the purpose of conciseness in the present discussion, the writer will restrict himself to a description of the main factors favoring increased plasma filtration.

Local vasodilatation and increased capillary pressure. Krogh (1922) assumed that the increased filtration through the capillary wall following the use of urethane was referable to the stretched endothelium of the dilated capillary. Landis (1927*a*) demonstrated by intravascular injection of dyes that there was no measurable increase in permeability with dilatation of the capillary. The passage of fluid seemed to be primarily referable to the level of intracapillary pressure. This investigator regards the effect [illegible] as [illegible].

administration, and measurement of the rate at which it disappears from the blood is used as a test of liver function (King and Aitken, 1940), and of thyrotoxicosis (Barnes and King, 1942).

PRINCIPLE

The glucose can be completely removed from diluted blood by fermentation with washed baker's yeast. Galactose remains unattacked and can be estimated in the deproteinized filtrate of the blood by the copper reduction method used in the estimation of blood glucose.

METHOD

Fresh baker's yeast (2 g.) is well washed by shaking with distilled water (10 ml.) in a centrifuge tube. The yeast is spun down; stirred up with a fresh 10 ml. of water, well shaken, and again centrifuged. The washing is repeated a third time. The yeast is finally stirred and shaken with 10 ml. of isotonic sodium sulphate and is ready for use.

The blood sample (0·2 ml.) is added to 2·5 ml. of a mixture of 22 ml. of isotonic sodium sulphate and 3 ml. of 10 per cent. sodium tungstate in a centrifuge tube. One ml. of yeast suspension is added. The contents of the tube are thoroughly mixed with the aid of a glass rod, and the tube is then incubated in a water thermostat at 37° C. for 15 minutes.

The proteins are precipitated by the addition of 0·3 ml. of 7 per cent. copper sulphate. The proteins and the yeast are filtered off or centrifuged (for 5 min.) and the galactose determined in 2 ml. of the supernatant fluid (equivalent to 0·1 ml. of blood) by the titration method described for blood glucose.

A blank determination consisting of 2·7 ml. of isotonic sulphate-tungstate and 1 ml. of yeast suspension is carried through at the same time as the other tests. The blank determination on the yeast should give the same figure in ml. of thiosulphate as is given by the blank determination with water, which is always carried out in the sugar

its permeability or capacity for passing fluids. Cohnheim called attention to this phenomenon about fifty years ago. Landis (1927*a*) pointed out that capillaries injured by alcohol and mercuric chloride *appear to be both permeable to the plasma colloids and approximately seven times more permeable to fluids than the normal capillary wall.*

The extent of injury to the capillary wall can be demonstrated in the early phase of the inflammatory reaction by a variety of means. For instance, graphite particles that normally fail to pass through the endothelial membrane readily do so in the early stage of the inflammatory process (Menkin, 1931*b*, see Fig. 1). Another convenient method

FIG. 1. Camera lucida drawing, showing passage of graphite particles through capillary walls into area of inflammation induced by croton oil (about 6 hours duration). Magnified approximately 970 X. (From Menkin, *J Exp. Med.*, 1931, *53*, 647.)

of demonstrating the alteration in the permeability of the endothelial wall in injury is with the use of intravenously injected dyes. Trypan blue and other dyes introduced into the circulating blood promptly accumulate in an inflamed area. The localization of vital dyes in areas of inflammation had been demonstrated by several investigators. MacCurdy and Evans (1912) pointed out that the normal brain and cord always remain free from dye injected intravenously but that areas of damage, such as softening or inflammation, *become stained deeply.* Bowman, Winternitz, and Evans (1912) found that trypan blue injected intravenously stains tubercles in experimental tuberculosis. Sub-

METHOD

This method is adapted from the procedure of Bratton and Marshall (1939). 0·2 ml. of blood is added to 3·2 ml. of water or isotonic sodium sulphate; 0·6 ml. of 25 per cent. trichloroacetic acid is added. The mixture is vigorously shaken, and filtered or centrifuged; 2 ml. of the filtrate ($\equiv$ 0·1 ml. of blood) are transferred to a test-tube and 1 drop of sodium nitrite solution added. The tube is shaken and left for 3 min.; 1 ml. of ammonium sulphamate solution is added and the mixture left for 2 min. with occasional shaking. Two ml. of naphthyl ethylene diamine solution are now added and the mixture shaken. The coloured solution is compared with a standard prepared in the same way from 2 ml. of standard solution ($\equiv$ 0·004 mg. sulphanilamide). The use of a yellow-green light filter (e.g., Ilford spectral yellow-green) facilitates the comparison.

Total sulphanilamide is determined by heating 2 ml. of filtrate with 0·5 ml. of N-hydrochloric acid in a 5 ml. volumetric flask in a boiling-water bath for 1 hour. The cooled contents of the flask are then treated with sodium nitrite, &c. as in the procedure for free sulphanilamide, and the volume adjusted to 5 ml. with water.

CALCULATION

$$\text{Blood sulphanilamide}^{*} \begin{cases} = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 0{\cdot}004 \times \dfrac{100}{0{\cdot}1} \\[2ex] = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 4 \end{cases}$$

* mg. per 100 ml. blood.

If the colour of the test is more than twice as strong as that of the standard, the determination should be repeated with 1 ml. of filtrate plus 1 ml. of water.

If sulphapyridine or sulphathiazole is determined by the above procedure, and with a sulphanilamide standard, the value obtained is multiplied by 1·4 to give the mg. sulpha-

area when the dye has previously been injected into the blood stream. Menkin (1929) demonstrated that trypan blue injected into the circulating blood enters rapidly the

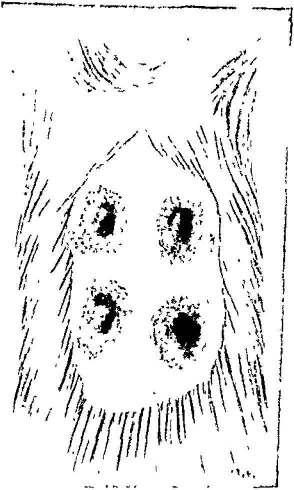


FIG. 2. The accumulation of trypan blue in areas of inflammation when the dye was injected into the circulating blood stream The areas of inflammation, which were of about four hours' duration, were induced in the skin of the abdomen of a rabbit by injection of concentrated broth The dye was injected intravenously fifteen minutes after the injection of the irritant.

site of inflammation and is fixed there, so that the tissues are deeply stained (Fig. 2). Furthermore, trypan blue injected directly into the site of inflammation in the subcutane-

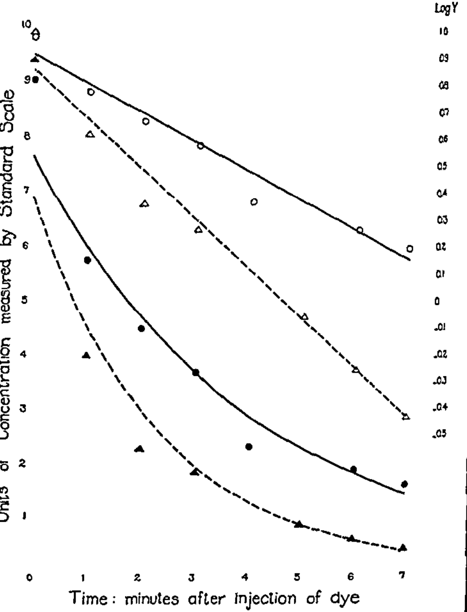
the oxygen-carrying power of blood. These yield valuable information concerning one important function of the blood, but they may provide a false picture of the pigment metabolism, since they take no account of inactive haemoglobin which may be capable of regeneration to the active form. They may imply the presence of anaemia where none exists. For this reason it has been deemed preferable (Clegg and King, 1942) to utilize the iron content of blood as a measure of its haemoglobin content. Blood contains about 50 mg. of iron per 100 ml. and almost all of this is present in the form of haemoglobin. Only a fraction of a mg. of non-haemoglobin iron is present in normal blood and this is largely confined to the plasma. Since all forms of haemoglobin contain iron in the same proportion, its estimation serves to give the total haemoglobin content of the blood.

Authorities differ as to the percentage of haemoglobin in blood which they regard as a normal figure. The three most commonly used are given in Table 3. There is an increasing body of evidence to show that Haldane's (1900) figure of 13.8 g. of haemoglobin is too low and that the Haden (1922) value of 15·6 g. per 100 ml. (20·9 ml. O_2) is a more normal figure (cf., Peters and Van Slyke, 1932; Wardlaw, 1941). This is the figure we have used and which has seemed adequately to represent our normal healthy cases; it has given colour indices averaging unity for healthy persons and treated anaemias.

TABLE 3.—*Concentrations of "Normal" Blood Standards*

	Haldane	Haden	Sahli
g. haemoglobin per 100 ml.	13·8	15·6	17·2
O_2-capacity (ml. per 100 ml.)	18·5	20·9	23·0
mg. Fe per 100 ml.	46·2	52·2	57·6

Four methods for estimating haemoglobin are given. The alkaline and the cyan-haematin methods estimate the total haemoglobin. The cyan-methaemoglobin method estimates oxidized and reduced haemoglobin, carboxyhaemoglobin and



● observed normal values
▲ observed values, inflamed mesentery
— concentration of dye in capillaries of normal mesentery (calculated)
--- concentration of dye in capillaries of inflamed mesentery (calculated)
○ logarithm of observed normal values
△ logarithm of observed values, inflamed mesentery

FIG. 3. The change in concentration of trypan blue in the capillaries of the mesentery. (From Menkin and Menkin, *J. Exp. Med*, 1930, *51*, 285.)

thereafter be used as a standard. Grey screens are mounted in optical glass and are procurable in any size and shape to fit any instrument.

Colorimetric. The haemin standard recommended by Clegg and King (1942) may be used interchangeably with a neutral grey screen for the comparisons in the alkaline haematin method. It is convenient to standardize a grey screen in combination with an Ilford mercury green filter in a Duboscq, or with a Chance green filter in a photoelectric colorimeter, by reference to a standard haemin solution. The haemin used by us for all standard solutions is British Drug Houses' crystalline haemin. 79·4 mg. of pure haemin (8·57 per cent. Fe) dissolved in 1 litre of N/10 NaOH gives the same intensity of colour as blood of 15·6 g. haemoglobin per 100 ml. when diluted 1 in 100 in soda, and compared, using a green light filter. Samples of haemin of other than theoretical iron content should be used proportionately, e.g. 81 mg. of B.D.H., 8·41 per cent. Fe haemin ($79 \cdot 4 \times 8 \cdot 57/8 \cdot 41 = 81$). New standards should be prepared at regular intervals—e.g. of two or three months.

For the cyan-haematin method a standard solution of 30·4 mg. of pure haemin of 8·57 per cent. Fe (31 mg. haemin of 8·41 per cent. Fe) in 1 litre of 1 per cent. sodium cyanide gives the same colour as 15·6 g. of haemoglobin treated first with hydrochloric acid and then with excess sodium cyanide, at a final dilution of 1 in 200 (King & Gilchrist, 1947).

PROCEDURES

Cyan Haematin. *Macro Method.* 0·5 ml. of blood are treated in a 100 ml. flask with 75 ml. N/10 HCl and left till the transformation to acid haematin appears to be complete, e.g. 5 to 10 minutes. Thereupon 20 ml. of 5 per cent. NaCN solution are added and water to the mark. The colour is read against the cyan-haematin standard with a green light filter.

Micro Method. 0·05 ml. blood are added to 4·95 ml. N/10 HCl, left for 5 to 10 minutes, and then 5 ml. of 2 per cent. NaCN added.

Alkaline Haematin. *Method 1.* 0·05 ml. of blood is diluted with 4·95 ml. of N/10 NaOH, heated in a boiling-water

CHAPTER IV

MECHANISM OF INCREASED CAPILLARY PERMEABILITY

The precise mechanism involved in the initial augmentation of capillary permeability in injury is obviously of considerable importance for an adequate understanding of the subsequent sequences in the development of an inflammatory reaction. In 1923 Ebbecke postulated that a substance is formed by irritated epithelium which on diffusing to the cutis dilates the capillaries and smallest arterioles. Subsequently Lewis (1927) postulated the development of a type reaction primarily referable to a chemical H substance liberated from injured tissue. The H substance is presumably histamine or a substance having similar properties and therefore resembling it closely. According to Lewis and Grant (1924) the type reaction elicited by the cutaneous injection of histamine manifests itself in three ways: a, a local vasodilatation of capillaries, venules, and arterioles by direct action; b, a widespread dilatation of outlying arterioles resulting from a local reflex; and c, an increase in the permeability of the minute vessels by direct action. This type reaction leads to local edema of the skin.

The conclusions of Lewis and Grant (1924) appear to be based largely on an analogy of the type reaction obtained by histamine with that of a variety of other injurious agents. When more direct tests were performed on the effect of the skin wheal fluid on the contraction of the guinea pig uterus, they were unable to obtain any evidence that histamine was liberated in larger quantities from injured tissue than was found in normal plasma.

Krogh (1929) accepts unreservedly the view first foreshadowed by the work of Ebbecke (1923), and later chiefly

optical density of any screen supplied may not be quite that which was ordered, but the density is always stated exactly.

With Duboscq colorimeters the screen (1 in. diameter) is placed on the left-hand rack in place of the standard cup, and the rack adjusted so that the screen is against the bottom of the plunger. The green light filter ($\frac{3}{4}$ in. diameter) is placed over the eye-piece. The coloured solution is placed in the cup on the right-hand side, and its depth is adjusted until a match is secured. Several readings are made and the average depth in mm. recorded.

In Table 4 are given the readings for test solutions prepared from six normal bloods according to the three colorimetric methods, and read against the grey screen on three different colorimeters of the makes most commonly in use. The same grey screen was used, but different spectrum green light filters. The same readings were obtained with all three instruments for any blood treated by any one method. The readings have all been calculated to a common basis—i.e., the depth in mm. at which the bloods, whose O_2-capacities had been accurately determined by Van Slyke, would match the grey screen when diluted to a haemoglobin content of 15·6 g. corresponding to 20·9 ml. O_2.

TABLE 4.—*Ilford Neutral Grey Screen Equivalents of Colorimetric Haemoglobin Methods*

(Duboscq colorimeters, 0 57 density grey screen and Ilford spectrum green filter; average readings for 10 bloods.)

	Alk. haematin			Cyanmet-Hb			Carboxy-Hb		
	1	2	3	1	2	3	1	2	3
Av. for each colorimeter (mm). . .	10·05	10·00	9·88	14 57	14 53	14·70	14 73	14 77	14·78
Av. for all colorimeters (mm). .		9·98			14 60			14·76	
Coefficient of variation (%) .		3·05			1·95			2 48	
Equiv. of 0·50 density grey screen (mm).*		$9\ 98 \times \frac{0{\cdot}50}{0{\cdot}57} = 8\ 75$			$14{\cdot}60 \times \frac{0{\cdot}50}{0\ 57} = 12\ 8$			$14\ 76 \times \frac{0{\cdot}50}{0{\cdot}57} = 12{\cdot}95$	

1. Bausch and Lomb colorimeter, macro cups and plungers.
2. Bausch and Lomb biological type colorimeter, micro cups and plungers.
3. Klett colorimeter, macro cups and plungers.

* A 0·50 density screen [illegible] spectrum green filter.

tion to injury. The contention raised by Rous and Gilding has been reinvestigated by Wayne (1931) who arrives at the conclusion that the vascular reactions resulting from injury to the skin are due to a liberated substance, either histamine or some substance closely allied to it. This worker answers the objection of Rous and Gilding by maintaining that the vasoconstrictor effect of Bier's spots can overcome the slight local vasodilatation produced by histamine, provided the latter is introduced electrophoretically into the skin in a diffuse manner and in low concentrations. The vasoconstriction will, however, not prevail over a more intense local vasodilatation induced by greater concentrations of histamine. He therefore concludes that the vessels of the skin, under the influence of either histamine or the H substance liberated by injury, behave similarly towards Bier's spots when the two are distributed in a comparable way throughout the tissue spaces.

A study was undertaken by Menkin (1936) in order to determine whether one or more substances could be obtained from inflammatory exudates which, when introduced into normal cutaneous tissue, would induce local vasodilatation and an increase in the permeability of the capillary wall. Furthermore, the properties of the active fractions which have been obtained from inflammatory exudates have been compared with histamine in an endeavor to test Lewis' hypothesis. In brief, the writer's observations indicate that a diffusible and apparently crystalline material capable of increasing capillary permeability is present in various types of inflammatory exudates. Its liberation and presence in exudates offers a reasonable explanation for the mechanism of increased permeability of small vessels in injured tissue. By appropriate tests this active principle has been shown to lack the properties characteristic of histamine (Menkin, 1936, 1938, 1939*a*). No concrete evidence was at first obtained to support Lewis' view of a detectable histamine-like substance in exudates or in their partially purified fractions. The possibility, however, that histamine exists in exudates

Chapter III

PROCEDURES FOR PLASMA

TAKING OF BLOOD FOR PLASMA

With a *dry* syringe blood is taken from an arm vein in the usual way. The arm should be warm; if a tourniquet is applied to distend the vein, it should be used immediately before the puncture is made and removed as soon as blood begins to flow. Five to ten ml. of blood are taken and allowed to flow gently into a test-tube or small jar which has been dried in an oven after the addition of 1 drop of a 30 % solution of potassium oxalate. The blood is thoroughly mixed, by repeated inversion (not violent shaking), with the oxalate. If the plasma is needed for CO_2-combining power or chloride analysis, the blood must be centrifuged immediately, and in any case the plasma should be separated and pipetted off from the cells as soon as possible, without chilling of the blood.

PLASMA PROTEINS

The total quantity of protein in blood plasma varies in normal individuals from approximately 6 to 8 g. per 100 ml. Plasma protein is divided into two main fractions: globulin and albumin. Globulin includes fibrinogen. Normally, the approximate amounts of the proteins in plasma are albumin 3·4–6·0 g. per 100 ml.; globulin (excluding fibrinogen) 1·5–3·0 g. per 100 ml.; fibrinogen 0·2–0·4 g. per 100 ml. Where there is decrease of plasma protein—e.g., through proteinuria or malnutrition—the albumin is chiefly affected, and there is often a reduction of the albumin-globulin ratio (normally 1·3–4·0). A reduction of this kind is characteristic of nephrosis. An increase in the globulin, especially fibrinogen, may accompany inflammatory conditions.

2

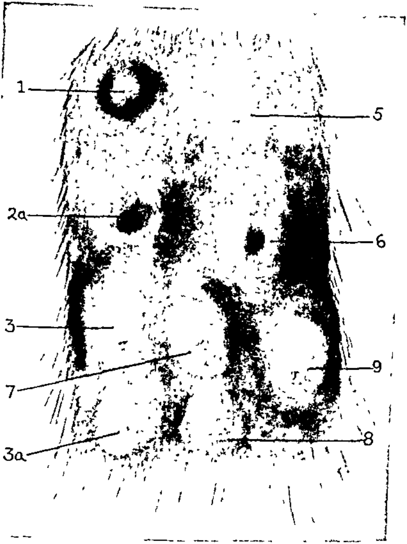


FIG. 4. The dermis of the abdomen of a rabbit treated with vari-

1. Cell-free exudate treated with saturated $(NH_4)_2SO_4$ and dialyzed. The dialysate was concentrated to about 1/17th of its original volume and inoculated in the skin. Note the considerable accumulation of the dye in the area.

2a The dialysate in area 1 was treated with 10 per cent $BaCl_2$ to precipitate out the $SO_4^{=}$ ions The supernatant fluid was diluted with an equal volume of phosphate buffer mixture (pH 7.43) and inoculated intracutaneously. The accumulation of dye is a conspicuous feature.

3 The protein material of the exudate remaining in the cellophane bag after dialysis. The dialysate of this sample was injected after

ammonium chloride solution (containing 0·01 mg. of nitrogen per ml.) and 3 ml. of Nessler's solution. A violet light filter may be used with advantage in this colorimetric comparison.

CALCULATION

$$\text{Total protein (A)*} \begin{cases} = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 0{\cdot}05 \times \dfrac{100}{0{\cdot}005} \times \dfrac{6{\cdot}25}{1000} \\ = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 6{\cdot}25 \end{cases}$$

* g. per 100 ml. of plasma.

NOTE.—If the highest accuracy in the total protein estimation is not necessary, the 0·5 ml. of diluted plasma may be digested with sulphuric acid without the preliminary precipitation of the protein with zinc hydroxide. The results will be approximately 0·15 per cent. too high. 0·15 per cent. protein corresponds to the average non-protein nitrogen equivalent of 24 mg. per 100 ml. of normal plasma. This simplification should only be used in cases where the N.P.N. is known not to be elevated.

(B) Fibrin. To 10 ml. (= 0·1 ml. of plasma) of the solution of plasma in isotonic sodium chloride, placed in a narrow tube, is added 0·2 ml. of calcium chloride solution. The mixture is kept at 37° C. until clotting occurs. The fibrin is carefully collected on a thin glass rod, pressed to remove liquid, washed with water, and dropped into a test-tube for digestion. This, and also the colorimetric estimation, is carried out exactly as in the case of total protein.

CALCULATION

$$\text{Fibrin (B)†} \begin{cases} = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 0{\cdot}05 \times \dfrac{100}{0{\cdot}1} \times \dfrac{6{\cdot}25}{1000} \\ = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 0{\cdot}3125 \end{cases}$$

† g. per 100 ml. of plasma.

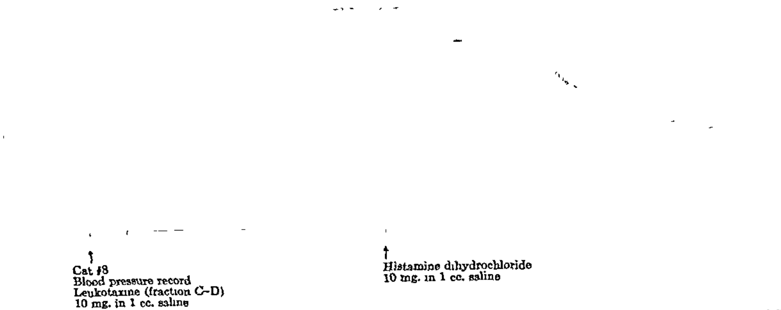


Fig. 6. The injection of 10 mg. of leukotaxine (fraction C–D) induced a transient *fall in pressure* in contrast to the progressive fall elicited by histamine dihydrochloride (10 mg.). (From Menkin and Kadish, *Am. J. Physiol.*, 1938, *124*, 524.)

PLASMA PROTEINS

Kjeldahl Method

PRINCIPLE

The plasma proteins are precipitated by molybdic acid and the precipitate is digested with sulphuric acid. By this process the protein nitrogen is turned into ammonium sulphate. The digested mixture is transferred to a distillation apparatus and the ammonia is liberated by adding an excess of sodium hydroxide. The ammonia is distilled by steam and is carried over into an excess of standard sulphuric acid solution. By titration of the excess of standard acid, that amount of it which has been neutralized by the ammonia is determined. From this the percentage of proteins is calculated.

The albumin, and by difference the globulin, is determined in a similar manner in the filtrate from a sample of plasma which has been treated with sodium sulphite solution. Treatment of the solution with sodium sulphite brings about precipitation of the globulin fraction and leaves the albumin in solution.

METHOD

Total Protein. 0·2 ml. plasma (from oxalated blood) is mixed with 5 ml. of water in a round-bottom Pyrex centrifuge tube. To this mixture are added 0·2 ml. of 7·5 per cent. sodium molybdate and 0·2 ml. of $\frac{2}{3}$ N-sulphuric acid. The tube is shaken and centrifuged for 5 min. The supernatant fluid is completely decanted off and the tube inverted and allowed to drain on a filter paper. Two ml. of 50 per cent. sulphuric acid (containing 1 per cent. selenium dioxide) are added, together with a small piece of porous pot. The mixture is heated on an electric coil heater or with a very small gas flame and is gently boiled until blackening occurs; thereafter the heating is continued for about 2 hours. If a condensate of selenium (reddish-brown deposit) forms at the side of the tube, it is returned to the body of the digestion mixture by gentle shaking.

inflammatory exudate.[1] Yet these investigators assume without any evidence, that this is presumably referable to the loose combination of histamine with proteins or by adsorption of colloids. In brief, their observations on the contraction of the isolated intestine of the guinea pig simply indicates that histamine probably exists in exudates, and can by appropriate extraction, be demonstrated. This, as mentioned above, has been surmised and known.

The permeability factor (i.e. leukotaxine) recovered and isolated from an exudate displays none of the specific properties of histamine. Yet leukotaxine is capable *per se* of reproducing the same type of reaction as the whole exudate. The mutual, non-specific properties of leukotaxine and histamine (e.g. dialyzing property, etc.), listed by Rocha e Silva and Bier seem quite insignificant for the most part, inasmuch as they are true of numerous other unrelated substances. These investigators have failed to repeat the work on the chemical extraction of leukotaxine from exudate. They have worked solely with whole exudates. They have thus been unable to establish the fact that leukotaxine is histamine; whereas the writer has definitely demonstrated that leukotaxine has a great many physiological and chemical properties differing from those of histamine (Menkin, 1936, 1938, 1939*a*).

A single very important concrete evidence which nullifies the contention of these investigators is indicated by the following observation: A sample of leukotaxine, capable *per se* of actively inducing increased capillary permeability and leukocytic migration is extracted for the presence of histamine by the method of Barsoum and Gaddum (1935). According to the contention of the Brazilian workers (1938), by this procedure, the presence of any depressing impurities in leukotaxine, capable *per se* of overshadowing the contractile effect on the intestine of histamine, would thus be eliminated. The final extracted material not only fails to

[1] This is generally found to be the case even when the concentration of histamine is as low as 1 500,000 (See footnote, page 27.)

process is repeated two or three times to ensure thorough cleansing of the distillation chamber. For the precautions which should be observed in performing the micro-Kjeldahl procedure, Pregl's " Quantitative Organic Microanalysis," 3rd ed., p. 88, should be consulted.

CALCULATION

1 ml. N/70 H_2SO_4 = 0·2 mg. of nitrogen

$$\text{Total Protein}^{*} \begin{cases} = \text{ml. N/70 } H_2SO_4\dagger \times 0\cdot 2 \times \dfrac{100}{0\cdot 2} \times \dfrac{6\cdot 25}{1000} \\ = \text{ml. N/70 } H_2SO_4 \times 0\cdot 625 \end{cases}$$

* g. per 100 ml. of plasma.
† neutralized by the ammonia.

Albumin. 0·5 ml. of plasma and a small drop of caprylic alcohol are placed in a 10 ml. volumetric flask or cylinder and 42 per cent. sodium sulphite solution is added to the mark. The mixture is well mixed and allowed to stand for 15 minutes, when it is filtered through a Whatman No. 32 or 42 filter paper. Five ml. of filtrate (= 0·25 ml. of plasma) are transferred to a round bottom Pyrex centrifuge tube. A drop of caprylic alcohol and 1 ml. of 50 per cent. sulphuric acid are added. The tube is shaken to drive off the SO_2 liberated from the sodium sulphite by the sulphuric acid. 0·5 ml. of 7·5 per cent. sodium molybdate is added to precipitate the albumin and the tube is shaken and centrifuged. The supernatant fluid is carefully decanted off and the tube drained. The subsequent analysis is carried out exactly as described for total protein.

CALCULATION

$$\text{Albumin}\ddagger \begin{cases} = \text{ml. N/70 } H_2SO_4\S \times 0\cdot 2 \times \dfrac{100}{0\cdot 25} \times \dfrac{6\cdot 25}{1000} \\ = \text{ml. N/70 } H_2SO_4 \times 0\cdot 5. \end{cases}$$

‡ g. per 100 ml. of plasma.
§ neutralized by the ammonia.

well and filtered after 5 minutes; 0·2 ml. of the filtrate ($\equiv$ 0·05 ml. plasma) is transferred to a centrifuge tube containing 1 ml. of absolute alcohol and 0·4 ml. of zinc uranyl acetate reagent. The contents are mixed and kept in the ice-box overnight;* they are then centrifuged for 15 minutes. The supernatant solution is decanted, the tube allowed to drain on a filter paper for 10 minutes, and the lip dried; 5 ml. of absolute alcohol saturated with sodium zinc uranyl acetate are added; the contents are mixed, by rotating the tube, centrifuged for 15 minutes and drained as before. The precipitate is then dissolved in 10 ml. of dilute acetic acid; 0·25 ml. of potassium ferrocyanide solution is added and after mixing the tube is allowed to stand in the dark for 5 minutes. The coloured solution is compared with that produced from a standard sodium chloride solution, 0·2 ml. ($\equiv$ 0·15 mg. Na) of which has been treated simultaneously in the same way as the deproteinized plasma. (Colours must be read within 15 minutes or a clouding may occur. Green light filter.)

CALCULATION

$$\text{Plasma sodium}\dagger \begin{cases} = \dfrac{\text{Reading of standard}}{\text{Reading of unknown}} \times 0{\cdot}15 \times \dfrac{100}{0{\cdot}05} \\[2ex] = \dfrac{\text{Reading of standard}}{\text{Reading of unknown}} \times 300 \end{cases}$$

† mg. per 100 ml. of plasma.

SOLUTIONS

Standard NaCl (containing 0·75 mg. Na per ml.).—191 mg. of analytical dry sodium chloride dissolved in 100 ml. in water in a volumetric flask.

Trichloroacetic Acid.—Seven g. per 100 ml. in water.

Zinc Uranyl Acetate Reagent.—Twenty g. of uranyl acetate, $UO_2(CH_3COO)_2.2H_2O$, 60 g. of zinc acetate, $Zn(CH_3COO)_2$.

* It is not possible to obtain complete precipitation of the sodium zinc uranyl acetate in a short time. A 2 hr. precipitation may be used, however, although the results will be less accurate.

TABLE I

Scheme of Extraction of Leukotaxine
(Permeability Factor)

- Exudate
  - Cell-free exudate
    - Pyridine or dioxane
      - Precipitate
      - Supernatant
        - Acetone
          - Precipitate (inactive or slightly active)
          - Supernatant
            - Active factor separates at $-20°$ C. (Fraction A) (5) *
            - Evaporated to dryness *in vacuo*
              - Active crystalline material
                - Prolonged extraction with butyl alcohol
                  - Active factor separates at $-20°$ C. (Fraction C) (9) *
                  - Supernatant evaporated to dryness *in vacuo*
                    - Stable active crystalline material with tarry admixture (Fraction C-D) (4) *
                      - Petroleum ether
                        - Precipitate dried *in vacuo*
                          - Relatively pure active material taken up in water (unstable)
                            - (Fraction M) (1) *
                      - Dilute HNO_3 or glacial acetic acid
                        - NH_4OH
                          - Precipitate in H_2O
                            - Dried *in vacuo*
                              - Active crystalline material (Fraction E) (2) *
                            - Dialysis against H_2O (24 hours)
                              - Residual indiffusible material
                                - Evaporated to dryness *in vacuo*
                                  - Active material (Fraction O) (3) *
                      - Saturated $(NH_4)_2SO_4$
                        - Dialysis against H_2O
                          - Residual material
                            - Evaporated to dryness *in vacuo*
                              - Moderately active material (Fraction N) (8) *
            - Dialysis against distilled water (2 to 3 hours)
              - Diffusate
                - Evaporated to dryness
                  - Water-soluble active crystalline material (6) *
              - Residual material
                - Evaporated to dryness
                  - Water-soluble active crystalline material (7) *

* The final fractions (6) and (7) reveal considerable inorganic impurities admixed with the active crystalline material Fractions (1), (2), (3), (8), (9), and to some extent (4) and (5) represent leukotaxine or the permeability factor probably in a relatively purer form In the latter state, it is essentially insoluble in water

matches the colour obtained when 0·1 mg. of bilirubin is treated with the diazo reagent in a final volume of 25 ml.

METHOD

One ml. of plasma is treated in a centrifuge tube with 0·5 ml. of diazo reagent,* 0·5 ml. of saturated ammonium sulphate, and finally 3 ml. of absolute ethyl alcohol. The mixture is stoppered, thoroughly mixed, allowed to stand for a few minutes, and filtered. Under these conditions the dilution of the plasma closely approximates to 1 in 4, allowance being made for the volume of the precipitate and for the change in volume when alcohol is added to water. The colour of the clear filtrate is compared with the standard mentioned above (= 0·1 mg. of bilirubin in a volume of 25 ml.) (Haslewood and King, 1937).

CALCULATION

$$\text{Bilirubin}\dagger \begin{cases} = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 0{\cdot}1 \times \dfrac{4}{25} \times \dfrac{100}{1} \\ = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 1{\cdot}6 \end{cases}$$

† mg. per 100 ml. of plasma.

It is frequently found that brownish or purplish tints produced in the reaction make colorimetric comparison with the artificial standard difficult. These extraneous colours (probably due to traces of substances other than bilirubin which react with the diazo reagent) may be eliminated by the use of a green light filter (see section on photometric measurement, p. 142). The green filter (Ilford spectrum green is an appropriate type) is placed over the eye-piece of the colorimeter and the reading made against the artificial standard. The adjustment is then made in a green field whose two halves are of exactly the same quality of colour and differ only in intensity. The reading is taken in the usual way.

* If the diazo reagent is carefully "layered" above the plasma, and the tube allowed to stand for a few moments, a positive "direct" reaction (if present) may be seen at the liquid junction.

doubly refractive granules, of needle-like or small rod-like crystals, or a mixture of both types of crystalline material. The crystals usually appeared as if they were bound together by the presence of an ill-defined, greyish, homogeneous matrix (Fig. 9). The crystalline material formed a cloudy suspension in water. When the latter was allowed to dry gradually in a desiccator, beautiful needle-like crystals occasionally separated out (Fig. 10). The similarity of the end-product attained by different analytical procedures strongly suggests that one is dealing with the same active substance. The crystalline material is extremely active in inducing in treated cutaneous areas of rabbits both increased capillary permeability and prompt leukocytic migration. In view of its additional chemotactic property, which is to be described at length in the next chapter, the writer has for the sake of convenience named this active substance *leukotaxine.* The test for chloride impurities is essentially negative. The sodium content is considerably reduced (6.4 per cent). The qualitative test for phosphorus is likewise negative. The available evidence indicates that this substance is in all probability the active permeability factor present in inflammatory exudates, isolated in a form which seems to be relatively free of any gross impurities.[1]

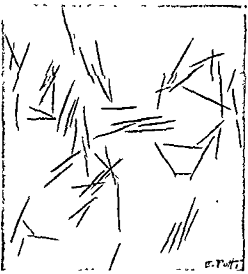


FIG. 10. High power drawing (about X 760) of needle-like crystals of the permeability factor. These crystals were obtained by gradually drying in a desiccator small amounts of the aqueous suspension of the material recovered after butyl alcohol purification (Fig 9). These crystals taken up in distilled water, induce prompt increased capillary permeability followed by considerable leukocytic migration. (From Menkin, *J. Exp Med*, 1938, *67*, 129.)

[1] Present day methods for testing the chemical purity of proteins or presumably

iodate, together with the silver chloride formed, is precipitated by the addition of acid, leaving in solution an amount of soluble iodate equivalent to the amount of chloride originally present. After the addition of potassium iodide, the amount of iodine set free from the soluble iodate is determined by titration with thiosulphate.

METHOD

0·2 ml. of plasma is added to 1 ml. of water. 0·4 ml. of zinc sulphate solution and 0·4 ml. of 0·5 N-sodium hydroxide are added and thoroughly mixed. The mixture is then centrifuged. One ml. of the supernatant liquid ($\equiv$ 0·1 ml. of plasma) is treated with silver iodate reagent (0·5 ml.) and after mixing, with 2 N-sulphuric acid (0·5 ml.). The mixture is shaken and filtered through a small fine paper. One ml. of filtrate ($\equiv$ 0·05 ml. of plasma) with the addition of 1 ml. of 1 per cent. potassium iodide, is titrated with 0·005 N-sodium thiosulphate, with starch as indicator (Haslewood and King, 1936).

CALCULATION

Chloride (as mg. NaCl per 100 ml. of blood) $= 97 \cdot 5 \times$ titre.

SOLUTIONS

Preparation of Silver Iodate Reagent.—Silver iodate is prepared by mixing equimolecular solutions of silver nitrate and potassium iodate. The precipitate is filtered, washed with distilled water, dried in vacuo, and preserved in the dark. Two g. of the dried solid are dissolved in 100 ml. N-ammonia. Both silver iodate and its ammoniacal solution appear to decompose slightly when kept, with liberation of soluble iodate. Immediately before a series of determinations, therefore, 5 ml. of the stock (2 per cent.) ammoniacal silver iodate are acidified with 2 N-sulphuric acid (5 ml.) and centrifuged. The supernatant fluid is discarded and the iodate redissolved in 5 ml. of fresh N-ammonia.

potency in the cruder fractions is not reduced by keeping it on ice for several weeks but occasionally its reaction becomes distinctly acid in contrast to an initial alkaline pH. The reactivity of the active factor does not seem to bear any direct relation to its effect on capillary permeability.

The contact of this substance for several hours with normal hydrochloric acid does not seem to affect its potency. Hydrolysis for about 20 hours in 5N HCl or 9.5N NaOH inactivates the material. Hydrolysis, however, with normal NaOH fails to either destroy its activity or to dissociate the permeability from the chemotactic factor by inactivation of the former (cf. this fact with the statement of Bier and Rocha e Silva, 1938).

4. *Effect of heat or cold.* The material is thermostable. When brought to 100° C. it is found still active. Heating it *in vacuo* at 85° C. for eleven hours has failed to reduce its activity. In a similar fashion, when exposed for several days at −20° C. the material retains full potency.

5. *Melting point.* In its present stage of purification the active material fails to show a sharp melting point. From about 200° C. leukotaxine (fraction C–D or E) begins to char; and at 300° C. it still has not melted. It is well known that a number of proteolytic compounds behave in similar fashion. Greenstein (1937) in his recent studies on peptide synthesis described a similar behavior in the case of the crystalline bisanhydro-l-cystinyl-l-cystine.

6. *Diffusibility.* The active permeability factor readily diffuses through a cellophane membrane upon dialysis of the untreated exudate (Menkin, 1936). As pointed out in Table I, the supernatant acetone fraction containing the active substance may be dialyzed for several hours against distilled water. Evaporation of both residual and diffusate fractions reveals the presence of the active crystalline material. The further purified fractions of leukotaxine, which are relatively insoluble in water, are probably more or less indiffusible when dialyzed against an aqueous medium.

in the combined form as sodium bicarbonate, and a determination of the amount of carbon dioxide which can be held by a sample of blood plasma gives a measure of the alkali present. The alkali of plasma is thus usually referred to in terms of its "CO_2-combining power." *In clinical conditions in which an* "acidosis" is present (e.g., diabetes, starvation, and severe nephritis) the production in the body of various acid substances results in part of the alkali of the plasma being combined with these other acid bodies. Less carbon dioxide is consequently held in combination by the plasma, and the "CO_2-combining power" is therefore reduced. In other clinical conditions involving the accumulation of excess alkali in the blood (e.g., alkali administration, intestinal obstruction, over-breathing) more carbon dioxide is held in combination in the plasma than is normally the case. This condition is usually known as one of "alkalosis."

PRINCIPLE

The carbon dioxide is liberated from its combination with alkali in the plasma by the addition of acid and the volume of gas evolved is measured in a special apparatus designed by Van Slyke. The volumetric method and apparatus introduced by Van Slyke and Cullen (1917) is described. This has been found more suitable for student use than the manometric apparatus of Van Slyke and Neill (1924). The latter should be used for work requiring the highest accuracy (cf., Peters and Van Slyke, 1932), but the simpler volumetric apparatus is adequate for all routine purposes.

METHOD

Five ml. of freshly drawn oxalated blood are centrifuged until the supernatant plasma is free of cells. About 2 ml. are transferred to a separatory funnel (about 250 ml.). By means of a short rubber tube, the stem of the funnel is connected to a bottle containing glass beads and a little water (see Fig. 1).

TABLE II

CORRELATION BETWEEN AMINO ACID LEVEL IN SERUM AND EXUDATE AND THE EFFECT OF THE LATTER ON CAPILLARY PERMEABILITY

Dog No	Duration of Inflammation	Amino Acid Nitrogen		Accumulation of Trypan Blue in Skin Areas Treated with	
		Blood Serum	Exudate	Blood Serum*	Exudate
	days	*mg./100cc.*	*mg./100cc.*		
1-04	1	5 35	6.4	+	++
	2	5.3	6.5	Trace	++
	3	2 9	3 3	0	+
	• 4	3.75	4 75	+	+++
Reinjected 1.5 cc. turpentine on 6th day					
	7	6.05	8.9	+ to ++	++++
	8	5.75	12.6	+	+++
	9	5.75	10 3	Faint trace	+++
	10	6.15	20 95	+	++++
1-05	1	7.8	7.6	Trace	Trace to +
	2	6.15	6 4	+	+
Reinjected 1 5 cc. turpentine	6	4.0	6.05	0	++++
	8	5.25	6 05	+	++
	9	4 3	5 35	+	+
1-06	1	7 6	6.1	+	+
	4	3 6	4.05	Trace	+ to ++
	5	8 2	12 25	Trace to +	+
1-03	5	6 4	9.2	0	++
Average		5 55	8 04		

* Cutaneous areas inoculated with serum invariably stained only the peripheral portion of the areas

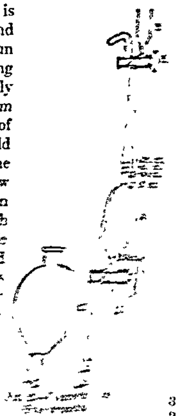
From Menkin, *J Exp. Med.*, 1938, *67*, 129.

protein. According to this author the ability of phagocytic cells to remove injurious material is dependent on the possession of proteolytic enzymes.

The end products of proteolytic breakdown in exudates have been studied by the writer (1938). The amino acid level was determined in samples removed from the inflamed pleural cavity of dogs. Parallel determinations were made on serum of blood withdrawn from the heart immediately following thoracentesis for the exudates. The colorimetric

into the top of the burette, air has leaked into the apparatus and the stop-cocks should be re-greased in order that they shall be airtight.

Distilled water (1 ml.) is added to the inlet chamber [illegible]. One ml. of plasma is removed from the separatory funnel and is run into the chamber (B) below the surface of the distilled water. A drop of caprylic alcohol is added as an anti-frothing agent and the contents of (B) are allowed to run into the burette by carefully opening the stop-cock (E) and very gradually lowering the mercury reservoir from its previous position at the height of (E). A small amount of water should be left filling the capillary at the bottom of (B). Stopcock (E) is now turned off, great care being taken that no air is allowed to pass through it into the burette. 0·5 ml. of lactic acid solution (1 vol. of conc. acid diluted to 10 vols. with water) is now placed in (B) and is carefully drawn into the burette in the manner already described, care being again taken to leave a small amount of liquid in the capillary at the bottom of (B) and admitting no air into the burette. The mercury reservoir is lowered until the mercury is at the 50 ml. mark and the stop-cock [illegible] of the burette and 50 ml. [illegible] vacuum and the carbon [illegible] from its combination [illegible] acid is seen to boil off from the [illegible] all the carbon dioxide [illegible] is gently shaken [illegible] carefully [illegible] apparatus, the [illegible] mixture very carefully [illegible]



3
2
10

0 to 15

[illegible] ributed in [illegible] of " organic [illegible] for the most [illegible] contained in the [illegible] gomyelin. There [illegible] in the cells as in the

monium sulfate and of 5 per cent phosphomolybdic acid to precipitate it, and finally its diffusibility through a cellophane membrane, all suggest that the active crystalline substance may well be a relatively simple intermediary product of protein catabolism. It appears unlikely that it is either a proteose, a peptone, or an amino acid. The available data are not inconsistent with the fact that this active substance can belong to the group of simple polypeptides. In this connection it is interesting to note that a proteolytic enzyme, incubated with an otherwise relatively inactive blood serum, favors the formation of products that increase vascular permeability (Menkin, 1937, 1938*b*). These findings offer an additional support concerning the general nature of the permeability factor.[1]

Observations on its incapacity to induce heart block in frogs (test of Parnas and Ostern, 1932) indicate that leukotaxine probably bears no relation to adenylic compounds (Fig. 11, Menkin and Kadish, 1938). Its failure to depress blood pressure and its inability to contract the isolated loop of guinea pig intestine likewise show that leukotaxine is evidently not related to acetylcholine. Further studies are being conducted, however, in an attempt to free the active crystalline substance completely of all impurities and to identify it chemically.

Duran-Reynals (1929) described a spreading factor present in extracts of certain tissues, particularly the testis. This agent facilitates the extension of India ink from the site of its cutaneous inoculation. He referred the phenomenon to an enhancement of tissue permeability by the organ extract employed. Recent unpublished observations indicate that the crystalline material recovered from exudates (leukotaxine) is probably unrelated to this spreading factor. The Duran-Reynals substance is relatively thermolabile and

[1] In this connection it is interesting to note that Duthie and Chain (*Brit. J. Exp. Path.*, 1939, *20*, 417) have recently confirmed in large part the studies of the writer on leukotaxine by obtaining a polypeptide from the peptic hydrolysate of blood fibrin which produces both increased permeability of blood vessels and leukocytic migration.

a very small amount of fluid at the bottom of the 50 ml. chamber. (F) is now turned round so as to be open to (C) and the mercury is allowed to run into the 50 ml. chamber and up into the graduated burette tube. The surface of the mercury in the reservoir is held level with the surface of the mercury in the burette tube, so that the carbon dioxide in the burette is now at a pressure equal to that of the atmosphere. The volume of the gas is read from the burette and is corrected to standard pressure and temperature. For this calculation the temperature and barometric pressure at the time of the experiment are, of course, necessary.

CALCULATION

The figure obtained, the ml. of carbon dioxide released from 1 ml. of plasma, is reduced by reference to the table to the number of ml. of carbon dioxide which would be liberated from 100 ml. of plasma at standard temperature and pressure. This is the "CO_2-combining power" of the plasma.

PHOSPHORUS: DISTRIBUTION IN BLOOD

The phosphorus of the blood is present partly as inorganic phosphate and partly in combination with several organic substances. It is distributed as follows:

	Whole blood	Plasma
	mg. P per 100 ml.	
Inorganic phosphate	2 to 3	2 to 3
Ester "	20 to 30	1 to 2
Lipide "	11 to 14	7 to 10
Nucleotide "	2 to 3	—
Total "	35 to 50	10 to 15

The inorganic phosphate is about equally distributed in cells and plasma. The ester phosphate consists of "organic salts" of phosphoric acid, which are present, for the most part, in the cells. Lipide phosphate is that contained in the phosphatides—lecithin, kephalin and sphingomyelin. There is about twice as much lipide phosphate in the cells as in the plasma.

be due to a local nervous mechanism. He referred the mechanism to an axone reflex located in the sensory fibers. These results have essentially been confirmed by Bardy (1915) and by Breslauer (1919).

Krogh (1920) observed that the application of iodine to the frog's tongue produced a violent contraction of the underlying muscles. The capillaries of the mucous membrane strongly dilated and the dilatation extended for a considerable distance. After the application of cocaine to paralyze the sensory nerves and the nerve endings, a drop of iodine failed to elicit the usual effect. When iodine was applied to the mucous membrane after section and degeneration of the lingual nerves, the affected capillaries became partially dilated over a localized area. Krogh (1922) concluded that, at least for certain substances, nervous reactions play some part in the initial inflammatory symptoms. The nerves which are responsible for the reaction are most likely sensory fibers that induce dilatation through local axone reflexes.

Ricker and Regendanz (1921), after studying the local action of a number of substances on the pancreas and conjunctiva of the rabbit, reject the views of Cohnheim in regard to the rôle of the vascular nervous system in inflammation. These investigators assume with, however, only meager evidence, an intricate arrangement of vasomotor nerves consisting of constrictor and dilator fibers supplying both arteries and capillaries. They contend that tissue alterations caused by the presence of an irritant are, in the last analysis, due to local circulatory disturbances arising through stimulation of involved vasomotor nerves.

More recently the studies of Inutsuka (1928) confirmed the earlier observations of Samuel (1890) and the later ones of Meltzer and Meltzer (1903) on the effect of sectioning the cervical or sympathetic nerves in enhancing exudation. This investigator demonstrated that excision of sympathetic ganglia induces marked exudation.

The stimulation of the peripheral ends of sensory nerves

METHOD

Two ml. of freshly drawn oxalated plasma or whole blood are diluted with 5 ml. of water and treated with 3 ml. of 25 per cent. trichloroacetic acid. The mixture is well shaken and is filtered after 5 minutes. Analyses for total "acid-soluble" and inorganic phosphate are carried out on the filtrate as below.

Total "Acid-soluble" Phosphate. Five ml. of the trichloroacetic acid filtrate from plasma (≡ 1 ml. plasma) or 0·5 ml. of filtrate from whole blood or cells (≡ 0·1 ml. blood) are measured into a 15 ml. volumetric flask of good acid-resistant glass, 1·2 ml. of 60 per cent. perchloric acid are added and a small piece of porous pot to prevent bumping. The contents of the flask are heated carefully with a micro-burner (using a very small flame) or on an electric heater. (Approximately 0·2 ml. of perchloric acid is lost in the heating.) As the contents of the tube become concentrated they turn brown and then, as the temperature rises and the acid begins to fume, they become colourless, the organic matter being completely oxidized in a few minutes. In some cases, where the amount of organic material is large and the oxidation slow, it may be necessary to add a drop of nitric acid or of 30 per cent. hydrogen peroxide; in this case it will be necessary to continue the heating for 3 or 4 minutes after the mixture has become colourless, in order to drive off the excess of these reagents. The cooled contents are diluted with about 10 ml. of water. One ml. of 5 per cent. molybdate and 0·5 ml. of reducing agent are added to the test. At the same time two standards are prepared from 5 ml. and 10 ml. of the standard solution (0·01 mg. P per ml.), 1 ml. of perchloric acid, 1 ml. of molybdate and 0·5 ml. of reducing agent. Test and standards are diluted to the mark, mixed, and the test read after 10 minutes against the appropriate standard, using a red or orange light filter (e.g., Ilford spectrum red or orange).

A method for the isolation and purification of this permeability factor, named leukotaxine, has been described. In its essential features, this consists of treating the exudate with pyridine or dioxane, followed by acetone. After separation of the protein fractions further purification can be obtained by prolonged extraction with butyl alcohol or by subjecting the acetone or butyl supernatant fractions to low temperature (−20° C.). The latter favors spontaneous separation of the active principle. Further impurities of a resinous nature can often be eliminated by various procedures (see Table I). The purified material seems to be a crystalline doubly refractive nitrogenous substance.

The factor is evidently not a protein, yet it contains amino and carboxyl groups. It gives a positive test for the presence of an indole nucleus in its structure (Adamkiewicz test). The active material in exudates is dialyzable; and it is precipitated by concentrated ammonium sulfate. The present evidence suggests that it is an intermediary breakdown product of protein metabolism, possibly belonging to the group of relatively simple polypeptides.

The active factor manifests no property in common with histamine or presumably with the hypothetical H substance assumed to be closely related to histamine. Observations do not substantiate Lewis' hypothesis of histamine or of its closely related H substance as the primary cause of increased capillary permeability in inflammation.

The relationship of local axone reflexes to increased capillary pressure, thereby favoring filtration into injured tissue, is pointed out.

is heated in a hot water bath until it is boiling. It is then cooled, made to volume with alcohol-ether and is thoroughly shaken. The mixture is filtered, and 10 ml. of filtrate are carefully evaporated to dryness (in two 5 ml. portions to minimize the chance of loss through frothing) in a 15 ml. flask. The phosphate is estimated by digestion with perchloric acid, as in the method for total acid-soluble phosphate.

Total Phosphate in Blood. The whole of the phosphate of the blood can be determined by the colorimetric method after destruction of the organic matter (proteins, fats, etc.) by boiling perchloric acid. For this purpose 0·1 ml. of blood, accurately measured, is digested and the determination carried out as in the total acid-soluble phosphate method.

SOLUTIONS

Trichloroacetic Acid Solution.—Twenty-five g. of best grade trichloroacetic acid are dissolved in water and made to 100 ml.

Ammonium Molybdate Solution.—Five g. ammonium molybdate dissolved in water and made to 100 ml.

Reducing Agent. 0·2 per cent. 1 : 2 : 4-aminonaphtholsulphonic acid in 12 per cent. sodium meta-bisulphite and 2·4 per cent. sodium sulphite (w/v).

The reducing agent may be prepared fresh for use from a tablet containing the correct amounts of 1 : 2 : 4-aminonaphtholsulphonic acid, sodium sulphite and sodium meta-bisulphite. A tablet is ground with 10 ml. of water, and the filtered solution is ready for use. (Obtainable from Messrs. Gallenkamp.)

Stock Standard Phosphate.—A stock solution is made by dissolving 2·194 g. of pure potassium dihydrogen phosphate (KH_2PO_4) in 500 ml. in water. This solution contains 1·0 mg. P per ml.

The Standard Solution is made by diluting 5 ml. of the stock solution to 500 ml. with water. This solution contains 0·01 mg. P per ml. Both solutions should be kept saturated with chloroform to prevent any bacterial growth, which might otherwise cause a loss of inorganic phosphate.

chemotactic influence. He found that glycine and leucine were definitely chemotactic whereas tyrosine and trimethylamine failed to attract leukocytes. Wolf studied the phenomenon of chemotaxis *in vitro* (1921). She found that the calcium ion was the only inorganic ion which *per se* was positively chemotactic. She reported that, to a certain extent, all amino acids and amines are likewise positively chemotactic.

Grand and Chambers (1937) recently reported that mechanically injured tissues liberate thermolabile substances which are positively chemotactic for polymorphonuclear leukocytes. McCutcheon and Dixon studied chemotropism of polymorphonuclear leukocytes *in vitro*. Their results led them to conclude that the chemotactic response is one of direction only. The rate of motion depends on other factors such as osmotic pressure of the medium, temperature, or the internal condition of the cell (1936). These workers found that whereas polymorphonuclear leukocytes were strongly attracted by staphylococci or tubercle bacilli, lymphocytes failed to exhibit any chemotropism (1935). Very careful observations on the emigration of leukocytes in the living animal (amphibian larvae and rabbits) were recorded by Clark and his collaborators (1920, 1936). In general these investigators noted that the emigration of leukocytes commences on the average about two and a half hours after the injection of the irritant (croton oil). The majority of the cells were polymorphonuclear leukocytes. Relatively fewer monocytes or lymphocytes migrated through the endothelial wall. Furthermore, the period of emigration lasted only several hours. After a while the endothelial wall seemed to undergo a reversal in consistency which prevented further migration. Strong chemotactic response by polymorphonuclear leukocytes was not necessarily accompanied by active phagocytosis on the part of these cells.

The mechanism of the migration of leukocytes to an area of injury has not yet been satisfactorily explained. Nordmann and Rüther (1930) believe that the phenomenon is

phoric acid (such as phenyl phosphate)—under standard conditions. The amount of phosphate or phenol so liberated may be taken as the measure of the amount of enzyme present. The phenol is more easily determined than the phosphate, and three times as much phenol (by wt.) as phosphorus is set free. The hydrolysis is carried out at the optimum pH of 10 for 15 minutes. The results thus obtained agree very closely with those of the method of King and Armstrong (1934) of which this is a modification,* and with the method of Jenner and Kay (1932). The results are expressed in arbitrary " units " of phosphatase activity.

The " unit " of phosphatase is defined as the amount of the enzyme which will set free 1 mg. of phenol in the given time under the conditions of the test; and hence " units " per 100 ml. = mg. of phenol set free from the phenyl phosphate under the standard conditions.

METHOD

Test. In a conical centrifuge tube are placed 2 ml. of buffer and 2 ml. of substrate. The tube is allowed to remain in a water-bath at 37° C. for 3 minutes. Without removal of the tube from the bath, exactly 0·2 ml. of plasma (which must be cell-free) is added and mixed. The stoppered tube is allowed to remain in the bath exactly 15 minutes. At the end of this time 1·8 ml. of dilute Folin-Ciocalteau phenol reagent are added and the mixture centrifuged or filtered.

Control. In another tube are placed 2 ml. of buffer and 2 ml. of substrate. 1·8 ml. of dilute phenol reagent are added, followed by 0·2 ml. of plasma and the mixture centrifuged or filtered.

Four ml. of filtrate from the test and control solutions are pipetted into test-tubes. One ml. of 25 per cent. sodium carbonate is added and the tubes replaced in the water-bath for 5 minutes to bring up the colour.

Comparison. The solutions are compared in the colorimeter with a standard made up at the same time by taking 4 ml. of standard-phenol-solution-and-reagent and 1 ml. of 25 per cent. sodium carbonate. The test solution is placed

* The Bodansky unit is about one third the King-Armstrong unit.

(1928) reported that histamine fails to induce leukocytic migration. They, therefore, concluded that the release of histamine from injured tissue does not provide a complete explanation of the process of inflammation. Moon (1935) essentially confirmed these observations.

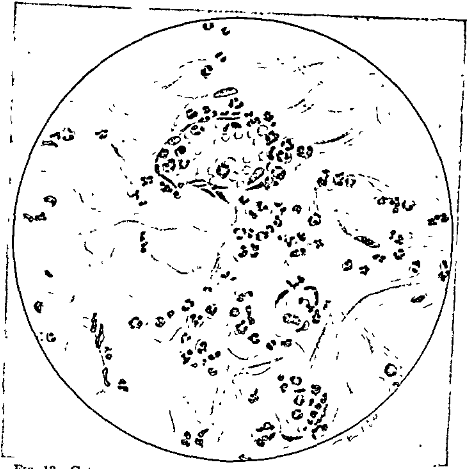


FIG. 13 Cutaneous vessel 41 minutes after the intradermal inoculation of purified leukotaxine [illegible] 145.)

Leukotaxine is concerned not merely with an alteration in the permeability of small vessels but also with the mechanism of leukocytic migration (Menkin, 1937*a*, 1938*c*). The introduction into the skin of rabbits of this active material in-

Substrate.—M/100 disodium phenyl phosphate. 2·18 g. dissolved in 1 litre in water. The solution should be brought quickly to the boil to destroy any organisms, cooled immediately and preserved with a little chloroform.

Phenol Reagent of Folin and Ciocalteau.—See p. 64; also Peters and Van Slyke's "Quantitative Clinical Chemistry," vol. ii, p. 655, and Beaumont and Dodd's "Recent Advances in Medicine," 8th ed., p. 403. This reagent is diluted 1 in 3.

Twenty-five per cent. Sodium Carbonate (w/v).—Twenty-five g. of anhydrous sodium carbonate are dissolved in warm water and made to 100 ml. This solution is preserved in a warm place, otherwise the sodium carbonate tends to crystallize out.

Stock Standard Phenol (100 mg. per 100 ml.)—One g. pure crystalline phenol is dissolved in, and made to 1 litre with 0·1 N-HCl.

Standard-Phenol-and-Reagent (1 mg. phenol per 100 ml.).—Five ml. of the stock standard phenol (100 mg. per 100 ml.) are accurately measured into a 500 ml. volumetric flask, 100 ml. of dilute (1 : 3) Folin-Ciocalteau reagent are added and water to the mark. This solution will keep at least six months, if preserved in the ice-chest.

ACID PHOSPHATASE

The prostate contains a very active phosphatase which, unlike the phosphatase of bone, intestine, kidney, etc., has its *p*H optimum at an acid reaction. To distinguish it from the better known "alkaline phosphatase," this enzyme is called "acid phosphatase." There is very little present normally in the blood, but in prostatic conditions, particularly in carcinoma of the prostate with secondaries in the bone, very large amounts may appear in the blood. These are derived in part from the prostate and in part from the secondary growths in the bone. There are normally 1–3 arbitrary acid phosphatase units in 100 ml. of serum or plasma. In cases of carcinoma of the prostate with secondaries, values much greater than this and up to 30 units and more have been observed (cf., Gutman and Gutman, 1938).

inary enhanced capillary permeability. Strong irritants, such as aleuronat or turpentine, although producing a prompt increase in the permeability of capillaries, fail to induce a rapid chemotactic effect (Fig. 14).

Furthermore, *leukotaxine is per se* chemotactic as indicated by *in vitro* tests (Menkin, 1938 *a*). This is exemplified by the following techniques: (a) Utilizing the method of

Fig. 15. The chemotactic effect of leukotaxine (fraction A, Table I, Chapter IV) studied *in vitro*. Supravitally stained polymorphonuclear leukocytes obtained from an exudate were placed on a slide containing a particle of *leukotaxine*. *Note the cellular aggregation and clustering around the material.* This drawing was made about three hours after the onset of the experiment.

Massart and Bordet (1891), the substance either in saline or in distilled water was introduced into glass capillary tubes. Each of the latter was then sealed at one end and introduced during ether anesthesia, into the peritoneal cavity of a rabbit. This animal had, several hours earlier, been intraperitoneally injected with a mixture of aleuronat and starch. After a

elevated. It may be preferable in these instances to use a somewhat longer incubation period, e.g., 3 hours. If this is done the mg. phenol liberated are of course divided by 3 to reduce the figure to 1 hour.

SOLUTIONS

See alkaline phosphatase for all solutions except the buffer. *Citric acid-sodium citrate buffer* pH 4·95 is prepared as follows:

Dissolve 21·0 g. of crystalline citric acid in water, add 188 ml. of N-NaOH, and make to 500 ml. The pH should be checked and adjusted to pH 4·9 if necessary, by dropwise addition of N-NaOH or N-HCl. This solution should be preserved with a few drops of chloroform and kept in the ice-chest.

N.B.—Acetate buffer may be used, but citrate is here adopted to make the method conform to Gutmans' 1940 procedure.

PLASMA AMYLASE

The determination of plasma amylase is of importance in the diagnosis of acute pancreatitis in which high values may be obtained. It has the advantage over the estimation of urinary diastase in that it is unnecessary to wait for the collection of a 24-hour specimen of urine. Normal values are given by Somogyi (1941) as 70–150 units per 100 ml.

PRINCIPLE

0·5 ml. of plasma is incubated at 37° with 1·5 mg. of starch and the time noted when the mixture no longer gives a blue colour with iodine solution.

The amylase activity is expressed in terms of "units" of amylase per 100 ml. The "unit" is defined as the amount of amylase which will destroy 1·5 mg. of starch in 8 minutes.*

* The unit is defined in this way so that the results become almost identical with those of Somogyi (1941) whose unit is defined in terms of the amount of reducing sugar liberated from starch by plasma under precisely stated conditions.

(b) Another type of evidence supporting the view that the substance in question is *per se* positively chemotactic for leukocytes was obtained by observing on a slide, within a *relatively short time, the collection and clustering around* particles of the active material of supravitally stained polymorphonuclear leukocytes obtained from an exudate (Fig. 15). Particles of carbon or reduced iron powder fail to induce any clustering of cells (Fig. 16). These observations indicate that the substance *per se* is positively chemotactic.

The available evidence supports the view that the permeability factor and the chemotactic factor are referable to one and the same crystalline substance. Two main considerations seem to support such a view.

1. The untreated inflammatory exudate contains both factors. Ultimate fractionation yields a relatively homogeneous crystalline end-product which still manifests both of the properties possessed by the original exudate. The analytical procedure has thus failed to dissociate the two factors. Although this type of evidence is strongly suggestive of a single substance, it is, however, to be borne in mind that only the certitude of complete chemical purity of the material can establish this as a definite fact.

2. *Is leukocytic migration referable to the initial increased* capillary permeability or do the evidences on hand point to a direct effect by the active crystalline substance? The latter interpretation seems to be favored in view of the observations cited above with the use of strong inflammatory irritants, *such as turpentine or aleuronat. These substances* are capable of inducing prompt filtration through the endothelial wall without at the same time inducing as rapid leukocytic migration as is obtained with the active material recovered from exudates. The chemotactic property of the active *crystalline substance is also in accord with the view* of a direct effect favoring the outward migration of polymorphonuclear cells.

In brief, the same two factors possessed by the untreated exudates have likewise been recovered in a crystalline sub-

healthy and showed no clinical signs of scurvy, had values ranging from 0·3 to 1·3 mg. with an average of 0·65. Only 2 per cent. of the cases were below 0·4 mg. (Young, Wood and King, 1943). Prunty and Vass (1943) considered the plasma ascorbic acid a reliable and satisfactory index of the nutritional state with respect to Vitamin C. The determination is more easily carried out than a saturation test (cf., urine ascorbic acid). A "state of saturation" is usually attained when the plasma ascorbic acid is 0·8 mg. per 100 ml. or greater.

PRINCIPLE

The oxidation-reduction dye dichlorophenol-indophenol is used to titrate a standard solution of pure ascorbic acid, which is prepared so as to be of about the same concentration as that of a deproteinized filtrate of blood plasma. The volumes of standard solution and of plasma filtrate used to decolorize a standard amount of the dye are then used to calculate the ascorbic acid concentration of the plasma.

METHOD

At least 5 ml. of oxalated blood are required. The plasma should not be separated until just before the preparation of the protein-free filtrate. Two ml. of plasma are diluted with 4 ml. of water and 4 ml. of 5 per cent. metaphosphoric acid, mixed and centrifuged. The clear supernatant fluid is then run (from a 5 ml. burette) into 0·05 ml. of dye until all trace of pink colour disappears. The titration should be made quickly so as to minimize the small amount of reduction of the dichlorophenol-indophenol which may take place due to traces of non-ascorbic acid reducing substances in the plasma filtrate. Any froth which might make the titration difficult can be cleared by touching with fine wire which has been dipped in caprylic alcohol.

To 2 ml. of the dilute standard solution are added 4 ml. of water and 4 ml. of 5 per cent. metaphosphoric acid. This is titrated against 0·05 ml. of the dye solution, conveniently contained in a conical centrifuge tube. The ascorbic acid solution is run into the dye from a 5 ml. burette until all trace

There is, however, some evidence that leukotaxine definitely affects normal cell division. Recent studies by the writer on the ova of sea urchins (Arbacia punctulata) indicate that this substance not only increases markedly the permeability of the cell to water but also influences its subsequent development. Fertilization of such leukotaxine-treated ova is followed by a retardation in the rate of cell division and by the appearance of atypical cleavage (Fig. 17). The sperm of sea

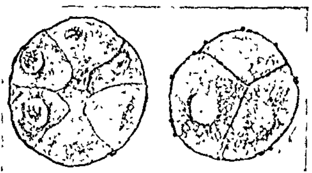


FIG 17. (Left) Normal cleavage of a fertilized ovum of Arbacia punctulata (Right) Note the unequal cleavage and the retarded development of a fertilized ovum of Arbacia punctulata which had been previously immersed in a medium containing leukotaxine. The interval after fertilization is the same in both ova

urchins exposed to leukotaxine are likewise inactivated (*Biol. Bull.*, 1939, *77*, 335). These facts, coupled with the effect on the permeability of the endothelial wall, therefore strongly suggest that leukotaxine induces some degree of cellular injury.

Furthermore, studies to be discussed in a subsequent section indicate that the leukocytosis which frequently accompanies severe inflammatory conditions is apparently not referable to the liberation of leukotaxine. This substance seems to be concerned with the mechanism of migration without directly affecting the number of leukocytes in the circulating blood. By such studies the writer has succeeded in dissociating the factors concerned with the mechanism of diapedesis from that which regulates the hematogenous picture of infectious processes (Menkin, 1938*c*, 1939*c*).

CHAPTER IV

PROCEDURES FOR SERUM

TAKING OF BLOOD FOR SERUM

About 10 ml. of blood are drawn (as for plasma) and allowed to flow gently into a clean dry vessel. This is then kept at room temperature, or in water at about 37° C., until the serum has separated. The blood must not be chilled, as this causes haemolysis as well as abnormal plasma/cell distribution of certain ions. The serum is poured from the clot into a centrifuge tube and is finally centrifuged.

SERUM CALCIUM

There are normally present about 10 mg. of calcium in 100 ml. of serum from freshly clotted blood. In hyperparathyroidism and conditions of generalized bone disease this amount may be raised by 2 or 3 mg. and occasionally in severe cases by 5 mg. or even more. In infantile tetany, or in tetany resulting from removal of part or all of the parathyroid glands, the serum calcium is lowered to 7 or 8 mg., and values as low as 6 mg. are sometimes encountered. Those cases of nephritis where an acidosis with raised blood phosphate is present, may also show a lowered serum calcium.

PRINCIPLE

The calcium is precipitated from diluted serum by oxalate, and the washed precipitate of calcium oxalate is titrated in acid solution with standard potassium permanganate. From the equation (see standard solutions) it can be calculated that 1 ml. of 0·01 N-permanganate is equivalent to 0·2 mg. of calcium.

rapid but it also does not seem to be primarily referable to the initial increase in vascular permeation. Furthermore leukotaxine is *per se* definitely chemotactic. Its rôle in explaining cellular migration at the site of inflammation is obviously of great significance. It is hoped that this substance may serve as an effective tool in yielding further information concerning the precise mechanism of diapedesis.

is a rise in Addison's disease, a small rise during attacks of bronchial asthma and in advanced renal disease, and a fall in familial periodic paralysis during the attacks of paralysis.

PRINCIPLE

The potassium is precipitated as cobalti-nitrite. The precipitate is washed, dissolved in hot water, and an excess of choline hydrochloride and sodium ferrocyanide is added. An emerald-green colour develops immediately, the depth of which is proportional to the amount of cobaltous salt present, and hence to the amount of potassium.

METHOD

This method is adapted from the procedure of Jacobs and Hoffman (1931). 0·5 ml. of serum (or of standard potassium solution) is placed in a conical centrifuge tube graduated at 4 ml., and 1 ml. of filtered Kramer and Tisdall's sodium cobalti-nitrite reagent is added slowly with constant shaking. After 45 minutes 1 ml. of water is added, and the contents are mixed and centrifuged at moderate speed for 15 minutes. The tube is then inverted and drained briefly on filter paper; 2 ml. of water are added down the sides of the tube without disturbing the precipitate. The tube is again centrifuged for 5 minutes, inverted and thoroughly drained. The precipitate is washed with 5 ml. of 70 per cent. alcohol, and centrifuged and drained. The alcohol is blown into the tube so as to agitate the precipitate. One ml. of water is added and the tube placed in a boiling water-bath until dissolution is complete.

In another graduated centrifuge tube is placed 1 ml. of the standard cobalt solution (≡ 0·1 mg. potassium); 0·5 ml. of choline chloride solution and 0·5 ml. of sodium ferrocyanide solution are added in that order to each tube and the volume made to 4 ml. The colours can be immediately compared in a colorimeter and are stable for several hours. (Orange or red light filter.)

cells through the capillary wall is, in his experience, infrequently seen. The recent work of Thomas (1938) indicates that several macrophages can readily originate *in situ* by budding from a single fibrocyte, this process being termed meroamitosis. Clark and his collaborators (1936) recently called attention to the possibility of artefacts. Injured or degenerating polymorphonuclear cells often round up and may present the appearance of mononuclear cells, round cells, or lymphocytes. This error can be readily avoided by utilizing the supravital technique on living cells as an additional check to the fixed preparations.

The orderly cytological sequence in the development of an inflammatory reaction was first pointed out by Borrel (1893) and then by Durham (1897) about forty years ago. The subsequent studies of Beattie (1903) extended considerably the original observations of Durham. This sequence is true of the majority of inflammatory reactions caused either by bacteria or by chemical irritants. The process has no direct reference to the actual migration of polymorphonuclear leukocytes into an area of injury. This phenomenon has already been dealt with in detail in the preceding chapter. It is noteworthy that during the first twenty-four hours after their inoculation into normal tissues both tubercle and typhoid bacilli produce the same type of cellular changes as do various forms of pyogenic bacteria such as *Staphylococcus aureus* (Borrel, 1893; Opie, 1910; Vorwald, 1932). The difference in the leukocytic response found with various types of inflammatory irritants seems therefore to be one of degree rather than of kind.

No adequate explanation has been offered for this fundamental process. A number of years ago various investigators, particularly Opie, studied the action of intracellular proteolytic enzymes from leukocytes of an inflammatory exudate (1905, 1906). Müller, and subsequently Opie, showed that polymorphonuclear leukocytes contain an intracellular enzyme that acts in a slightly alkaline or neutral medium, but is almost wholly inactive in an acid reaction (0.2 per cent

CHAPTER V

PROCEDURES FOR CEREBRO-SPINAL FLUID

The chemical determinations most frequently of value in the examination of cerebro-spinal fluid are those of protein, chlorides, sugar, calcium and urea. Lange's colloidal gold reaction is also of importance.

Globulin tests in normal fluid are negative. Total protein may be increased in many pathological conditions. Chlorides are lowered characteristically in meningitis, especially tubercular meningitis. Sugar is also lowered in meningitis. Calcium may be lowered in tetany, while the urea value closely parallels the level of blood urea.

TABLE 6.—*Composition of C.S.F.*

Constituent	*Normal range* (per 100 ml.)	Clinical conditions in which high values (unless otherwise stated) are found
Urea . . .	15–30 mg.	Increased in nitrogen retention.
Creatinine .	0·7–1·5 mg.	" " " "
Sugar . . .	60–100 mg.	Diabetes. Reduced in acute suppurative meningitis.
Chlorides (as NaCl)	700–740 mg.	Nephritis. Decreased in meningitis, particularly tuberculous meningitis.
Proteins (total) .	20–40 mg.	Meningitis. Syphilitic conditions. Froin's Syndrome.
Globulin {Pandy . Nonne-Apelt}	reactions negative.	...
CO_2-combining power	55–65 ml.	

PRINCIPLES OF METHODS

Total protein is determined by the sulphosalicylic acid method, with the use of permanent standards. The reactions of Nonne-Apelt and Pandy are used to test for globulin. Nonne-Apelt's test depends on the precipitation of globulin by

TABLE IV

THE HYDROGEN ION CONCENTRATION AND THE CYTOLOGICAL PICTURE IN ACUTE INFLAMMATION

Dog No	Interval between Injection of Irritant and Removal of Exudate	Differential Leukocyte Count of Inflammatory Exudate			pH of Inflammatory Exudate	Differential Leukocyte Count and pH of Blood			
		Polymorphonuclears	Lymphocytes	Mononuclear Phagocytes		pH	Polymorphonuclears	Lymphocytes	Mononuclears
	hrs. mins.	per cent	per cent	per cent			per cent	per cent	per cent
4	19 15	78 0	2 0	20 0	7.15				
	43 45	78 0	1 0	21.0	7 23				
	67 15	87.0	1 0	12 0	7.23				
	93 08	31 0	3 0	60 0	6 97				
	115 00	28 0	2.0	72 0	6.95	7.07	83 0	7.0	10 0
5	22 57	79 0	3.0	18 0	7.15				
	47 00	9.5	0 5	90 0	6 8				
	71·15	2.0	0 0	98.0	6.6	7.4	77.0	13 0	10 0
7	24.10	68.5	7.0	24.5	7.1				
	48·30	72 6	3 4	24 0	7.35				
	72.45	75 7	6 0	18.3	7.45				
8	24·50	90 0	0 5	9 5	7.23				
	47.35	87.0	2.7	10 3	7.13				
	71.15	63.25	0.5	36.25	6.78				
	100.05	59 0	1.0	40 0	6.98	7.1			
3	23 00	90 0	1.5	8 5	7 05				
	48 10	53 5	1.0	45 5	6.75				
	72:40	7 0	1 0	92.0	6 6				
	95 40	10 0	3 0	87 0	6 65	7.23	71.0	7.5	21.5
2	23·42	69 3	8.3	22 3	7.4				
	47 37	74 3	2 6	23.0	7.4				
	71 12	83 0	2 0	16.6	7.25				
	95 22	88 3	0 6	11.0	7 25				

CALCULATION

1 ml. of $\frac{M}{58 \cdot 5}$ silver nitrate ≡ 1 mg. of sodium chloride.

Hence,

C.S.F. chloride* = ml. of silver nitrate used × 100.

* mg. NaCl per 100 ml.

The adsorption indicator method (p. 46) may also be used for C.S.F. chloride.

LANGE'S COLLOIDAL GOLD REACTION

PRINCIPLE

This reaction depends on the fact that although normal cerebro-spinal fluid has no action on a particular colloidal gold solution, fluid from cases of syphilis, disseminated sclerosis, or meningitis may cause various degrees of precipitation of the gold at different dilutions of the C.S.F., which are fairly characteristic for each disease.

Typical responses are :—

Luetic . . .	0 1 3 4 3 2 1 0 0 0
Paretic . . .	5 5 5 4 3 2 1 0 0 0
Meningitic . . .	0 0 1 1 2 3 2 2 1 0

These figures serve to indicate the degrees of precipitation in tubes 1–10 in that order (see below).

A meningitic type of curve is found in all forms of coccal meningitis and tuberculous meningitis. A paretic type of curve is found in G.P.I., in tabes, in disseminated sclerosis and rarely in encephalitis lethargica. When a paretic curve occurs in association with a positive W.R. and is unaffected by antispecific treatment it is symptomatic of G.P.I. rather than tabes : when it occurs with a negative W.R. it is strongly suggestive of disseminated sclerosis. A luetic type of curve occurs in all forms of cerebral syphilis. It may also be found in disseminated sclerosis and is more common in encephalitis lethargica than the paretic type.

immediately or at least very soon followed by a fall in the percentage of polymorphonuclear leukocytes. By studying the hydrogen ion concentration one can fairly well predict the cytological picture in the exudate, and *vice versa*. The correlation is evidently very close. In Dogs 7, 2, and to some extent in Dog 10 the pH failed to become acid concomi-

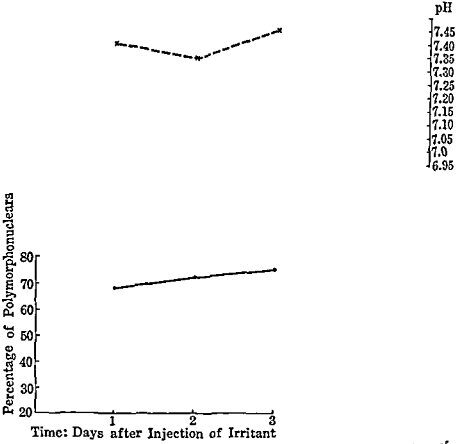


FIG. 19. The hydrogen ion concentration in relation to the percentage of polymorphonuclear leukocytes in pleural exudation from Dog 7. Note that the pH remains alkaline and that the percentage of polymorphonuclears maintains a high level throughout the duration of the experiment. (From Menkin, *Am. J. Path*, 1934, *10*, 193)

tantly with the progress of the inflammatory reaction. The counts correspondingly reveal a predominance in the percentage of polymorphonuclear cells throughout the period of the experiments (Fig. 19, Dog 7). In Dog 11 the per cent of polymorphonuclears appears surprisingly low for an inflam-

This preparation requires considerable practice. It is essential that all glassware used be perfectly clean, and that for all solutions, water twice distilled from glass should be used. A chromic acid mixture (20 g. of potassium dichromate dissolved in a minimum amount of water and the volume made to 1 litre with concentrated sulphuric acid) may be used for cleaning glass apparatus, which should be stored in the mixture when not in use.

The solutions required are potassium oxalate (1 g. per 100 ml.) and gold sodium chloride (1 g. of the salt $NaAuCl_4.3H_2O$ per 100 ml. of water).

(*b*) *Citrate Method.*—In a litre flask are placed 675 ml. water twice distilled from glass. The flask is closed with a ground-in reflux condenser and brought to the boil. Seven ml. *of 1 per cent. gold chloride solution are added, followed* by 17·5 ml. of 1 per cent. trisodium citrate solution. The boiling is continued for 15 min. The flame is removed and the mixture allowed to cool. The resulting solution is a clear sparkling red colour.

To standardize this solution, four 50 ml. samples are taken and 0·15 ml. of N/10 hydrochloric acid, 0·175 ml., 0·2 ml., and 0·225 ml. are added. These are put up against a known paretic C.S.F. The mixture is selected which gives the strongest reaction with a paretic fluid, yet does not give a reaction greater than 0110000000 with a normal fluid. The corresponding quantity of acid is added to the main bulk of solution, thoroughly agitating the mixture during the addition.

If a paretic C.S.F. is not available, a solution of 0·8 per *cent. washed haemolyzed sheep cells, used in the test like* a C.S.F., will give a paretic curve.

suggestive in answering this question. An examination of the results obtained in the case of some individual experiments points out that the increase in the hydrogen ion concentration evidently precedes the fall in the percentage of polymorphonuclear leukocytes. The point is exemplified in the case of Dog 13, Figure 20. For the first 2 days the pH was alkaline, 7.4 and 7.5 respectively. The percentage of polymorphonuclears was high, 87.5 and 77.5. On the 3rd day there was an abrupt fall in pH to 6.98. The percentage of polymorphonuclears, however, was still high, namely 74. On the 4th day the pH was lower than on the preceding day, namely 6.8. The exudate contained only 10 per cent of polymorphonuclears. Hence in this experiment the sharp rise in hydrogen ion concentration definitely preceded the fall in the percentage of polymorphonuclear leukocytes. The latter followed the decrease in alkalinity only after the lapse of a definite period. This is evidence that the fall in pH precedes the changes in the cytological picture. The hydrogen ion concentration may thus possibly be the regulating factor in determining the differential leukocyte formula of an exudate. In view of what is known of the mechanism of intracellular enzyme action in leukocytes, a physicochemical regulatory mechanism of this type would not be wholly unexpected. That the fall in pH seems to precede the drop in the percentage of polymorphonuclear leukocytes is quite evident from the above analysis. At the same time it is obvious on examining the data that this relation is not always evident. This seems to depend on the rapidity of the change in reaction. If the rise in hydrogen ion concentration is rapid and sharp the corresponding fall in the percentage of polymorphonuclears may occur so rapidly as to appear to be a parallel phenomenon (see Dog 15, Table IV). When the change in reaction proceeds very rapidly the exudate smears invariably reveal numerous degenerated, swollen, and vacuolated polymorphonuclear leukocytes containing characteristically fragmented and intensely stained nuclei. Such lethal effects accompanying an abrupt change in the reaction with

Benzidine Reaction

(*a*) A thin faecal suspension is made by shaking a small amount of faeces (about as big as a pea) with 5 ml. of water in a test-tube. The mixture is boiled to inactivate enzymes. (These, as well as blood, may give positive results, but unlike the active grouping of haemoglobin, they are destroyed by heat.) The extract is cooled; about 1 ml. of it, in another test-tube, is treated with 2 ml. of benzidine solution and 1 ml. of hydrogen peroxide solution. If the test is positive a blue colour develops at once.

(*b*) A little faeces is smeared on a glass slide, which is then placed on a boiling water-bath for 5 minutes. A mixture of benzidine and hydrogen peroxide solution (2 : 1) is dropped on the smear. A blue colour is indicative of a positive test.

Gum Guaiacum Reaction

One ml. of a boiled and cooled suspension of faeces, made as above, is mixed in a test-tube with 1 ml. of an alcoholic solution of guaiacum resin. 1 ml. of hydrogen peroxide is then added and the tube is well shaken, with further additions of alcohol to dissolve the precipitated resin. A blue colour indicates a positive test. This test may be modified, as above, for use with slides: in this case a clear mixture (approximately 2 : 1) of alcoholic resin and hydrogen peroxide is dropped on the dried faecal smear.

SOLUTIONS

Benzidine Solution.—Approximately 3 g. per 100 ml. made by shaking 3 g. of benzidine in cold glacial acetic acid (100 ml.) until solution is complete.

Hydrogen Peroxide Solution.—10 vols. (3 per cent.).

Gum Guaiacum in Alcohol ("Tincture of Guaiac").—Approximately 1 g. of resin is dissolved in 100 ml. of industrial spirit. The solution must be freshly prepared; it will keep for about a month.

ess. It becomes somewhat difficult to consider this state of affairs as mere coincidence. Secondly, the fact that by determining the pH of the exudate the character of the cytological picture could be fairly well predicted and *vice versa* seems to be definite evidence of some correlation between the pH and the cell count. In the third place, the fact that in a few instances when the shift from alkaline to acid took place rapidly the cell change, although delayed, nevertheless invariably followed appears to warrant the inference that if there is an interdependence it is the pH that conditions the cytological picture and not the reverse order of sequence. The observations seem therefore to support the conclusion that the differential leukocyte picture at a given time in the development of an inflammatory reaction is a function of the pH of the exudate.

The implications of this concept are obvious. It is possible that an understanding of the histological differences of various inflammatory lesions may be facilitated through a study of their respective hydrogen ion concentrations.

Opie (1910) pointed out that the studies on intracellular enzymes of leukocytes have served to explain many of the phenomena of resolution. Some of his earlier conclusions (1906*a*) on the solution of tissue with abscess deserve perhaps revision, in view of the writer's observations. Briefly stated, Opie's original experiments on abscess formation consisted in inducing a purulent exudation by the subcutaneous injection of turpentine. Four or 5 days later a large cavity distended with fairly thick purulent fluid was formed. The cells of this pus were separated from the serum by centrifugalization. To the cell-free pus serum, leucoprotease was added. This combination freely digested coagulated serum. On the other hand, the same polymorphonuclear enzyme in the presence of blood serum failed to digest materially the coagulated serum. From these facts Opie concluded that the anti-enzymatic action of a limited quantity of exuded serum is overcome by an increasing quantity of proteolytic enzyme set free by disintegration of polymorphonuclear leukocytes,

ferred to a 100 ml. glass stoppered cylinder. 10 ml. of water and 3 ml. conc. hydrochloric acid are added. The cylinder is placed in a hot water bath for 10 minutes.* After cooling to room temperature 50 ml. of ether are added and the contents thoroughly mixed by inverting the tube 60–80 times. By this means all the fat of the faeces should pass into the ether. The cylinder is left stoppered in a vertical position until the ether layer is completely separated. Twenty-five ml. of the ether extract are now removed and are placed in a weighed evaporating basin. The ether is removed by warming the dish on a water-bath or a hot plate and then placing in a vacuum desiccator containing a few lumps of paraffin wax in a beaker. The increase in weight in the dish represents the fat content of half the total ether extract and hence of 0·25 g. of dried faeces. The result is expressed as g. total fat per 100 g. dried faeces.

B. Neutral Fat plus Free Fatty Acids (unsoaped fat). An ether extraction of the faeces which have not been treated with hydrochloric acid will dissolve out the neutral fat and the free fatty acid without removing any of the fat present as soap. 0·5 g. of dried faeces is transferred to another 100 ml. glass-stoppered cylinder and 10 ml. of water are added without the hydrochloric acid used in the case of total fat. After warming for 10 minutes as in " A " the cylinder is cooled and 50 ml. of ether added. The ether soluble fat is extracted as before by thorough mixing of the contents of the tube. Twenty-five ml. of the extract are removed to a weighed dish and from the weight of the residue the g. of neutral fat plus free fatty acid in 0·25 g. of dried faeces is obtained.

C. Free Fatty Acid. The free fatty acids in the residue from (B) are determined by titration with N/10 sodium hydroxide in alcohol. The residue is dissolved in 10–20 ml. of absolute alcohol and a few drops of phenolphthalein added. The alcoholic sodium hydroxide is run in from a burette until the production of the first permanent pink colour. The

* Care must be taken to warm the cylinder gradually, otherwise it will crack.

acter of lymphoprotease and resembling the autolytic enzymes of tissues. In an endeavor to throw further light on this question the writer injected subcutaneously 1.5 cc. of turpentine into the right flank of a few dogs. Four days later a large subcutaneous abscess containing thick viscous pus resulted. The pH of this exudative material was definitely acid in reaction, approximately 6.6. This would support the contention that an excess of leucoprotease could not possibly be the important factor in the solution of tissues in such abscesses since this enzyme is active only in an alkaline or neutral medium.

Bayliss (1924), as a result of his studies on emulsin, expressed considerable doubt as to the actual existence of true anti-enzymes as follows: "Some of the effects described as being due to them are to be accounted for by changes of hydrogen ion concentration, others to adsorption of the enzyme by a colloid." Bayliss pointed out that in his emulsin experiments the effect was found to be due merely to diminution of the acidity of the solution. The inhibitory effect of the anti-enzyme disappeared when the solution was brought back to the initial value by the addition of acid phosphate. This idea may doubtless have considerable importance in revising our accepted concepts concerning the so-called "anti-enzymes" in inflammation, especially in view of the progressive increase in hydrogen ion concentration in such pathological areas. Nevertheless, although Bayliss may be correct as far as anti-enzymes are not comparable in regard to specificity to the true antibody, still by counteracting the effectiveness of the enzyme, whether by adsorption or by changes in the hydrogen ion concentration, the anti-enzymatic effect of serum on enzymes remains a fact.

The Mechanism of Local Acidosis in Inflammation

The mechanism conducing to local acidosis in inflammation is still somewhat problematical. The studies of Irisawa (1893) and of Ito (1916) have shown that lactic acid is a constant constituent of pus. Gessler (1932) has demon-

CHAPTER VII

URINE

Approximate Average Daily Composition of H

Nitrogenous Constituents—

Urea	30 g.	(as N, 14 g.)
Ammonia	0·8 g.	(as N, 0·7 g.
Creatinine	1·5 g.	(as N, 0·56 g
Hippuric Acid	0·5 g.	(as N, 0·04 g.
Amino-Acids		(as N, 1 g.)
Uric Acid	0·4 g.	(as N, 0·17 g.

Urochrome and other pigments.

Sulphur-containing Constituents—

Inorganic sulphates (as H_2SO_4), 1·8 g.
Ethereal sulphates, e.g., indican, 0·3 g.
" Neutral " sulphur compounds, e.g., NaCNS

Other Constituents—

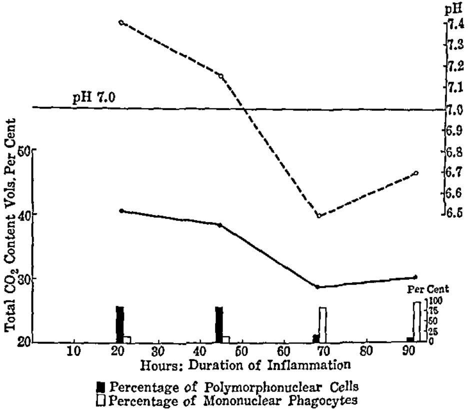
Organic
Oxalic acid
Carbonic acid · 0·02 g.
Aromatic oxyacids, e.g., *p*-hydroxyphenylaceti

Inorganic

Phosphate (as P)	1·7 g.
Chloride (as NaCl)	12 g.
" (as Cl)	7·3 g.
Sodium	10 g.
Potassium	2·5 g.
Calcium	0·2 g.
Magnesium	0·2 g.
Water	

The point is illustrated in Table V which summarizes a type experiment.

These same data are graphically presented in Figure 21. The interrelation between pH, CO_2 content, and the cytological picture is quite apparent. With the fall in pH there is a



a predominance of polymorphonuclear cells to a phase where mononuclear phagocytes constitute the chief element. (From Menkin and Warner, *Am J. Path.*, 1937, *13*, 25)

concomitant reduction in the local alkali reserve, as measured by the CO_2 content. Parallel with these changes in the acid-base equilibrium, the polymorphonuclear cells become displaced by mononuclear phagocytes. If the exudate remains alkaline throughout the period of the acute reaction, the CO_2 content likewise fails to be reduced. On the other hand, if a

(*b*) *Osazone Test.* To 5 ml. of the urine in a wide test-tube are added 0·2 g. of phenylhydrazine hydrochloride, 0·4 g. of sodium acetate crystals and 1 drop of glacial acetic acid. The mixture is heated for 45 minutes in a boiling water-bath, and is then filtered and allowed to cool slowly. Glucose and lactose each form characteristic yellow osazones, distinguishable by microscopic examination. Lactosazone forms globules or clusters of fine crystals resembling fluffy yellow balls. Glucosazone gives lath-shaped crystals.

ESTIMATION OF GLUCOSE

In diabetes mellitus it is frequently useful to determine the amount of sugar excreted in the urine. The dosage of insulin may be gauged by the daily excretion of sugar, the patient being under dietary control. The efficacy of the treatment may be most accurately determined by means of the estimation of the fasting blood sugar in conjunction with the above, but when facilities for the determination of blood sugar are not available it is possible to handle diabetes with reasonable success by the use of urine sugar estimations alone.

Meyer's Modification of Benedict's Method

The method affords a rapid and simple means for the estimation of sugar in urine.

Five ml. of Benedict's quantitative reagent together with 1 to 2 g. of sodium carbonate are placed in a large test-tube. About 5 ml. of distilled water are added to the mixture. The solution is brought to the boil over a small flame. The urine is added drop by drop from a 1 ml. graduated pipette. A few seconds' boiling is allowed between each addition of urine. The end point is recognized by the disappearance of the blue and green colour. Five ml. of Benedict's solution are completely reduced by 10 mg. of glucose, 11·6 mg. of galactose, or 13·4 mg. of lactose. Fresh batches of Benedict's reagent should be tested with 1 g./100 ml. solutions of pure glucose and lactose (also with galactose if the reagent is to be used for the galactose excretion test).

TABLE VI

The Relation of Lactic Acid Level to the Hydrogen Ion Concentration and to the Cytological Picture in Acute Inflammation

Dog No	Amount of Turpentine Injected	Total Duration of Inflammation	Sugar (mg per 100 cc)		Lactic Acid (mg per 100 cc)		pH		Cytology of Exudate	
	cc	hrs /mins	Blood	Exudate	Blood	Exudate	Blood	Exudate	Per Cent of Polymorphonuclears	Per Cent of Mononuclear Phagocytes
9–2	1 5	0 0	100.0				7 50		.	.
		19 10	88 1	96 0	10 8	54 6	7 45	7.43	Relatively acellular	
		47 10	111.9	57.9	13.0	27 0	7.48	7 33	85	15
	2 0 reinjected	68 0	145 5	116 8	16 0	27.8	7.48	7 33	74	26
		96 15	81 2	13 9	21 9	135 8	7 43	6 5	15	85 (few cells)
		115 0	138 6	6 4	38 4	248 6	7 43	6 5	2	98
		167 0	138 6	34.7	14 7	217 1	7 45	6 5	2	98
9–1	3 0	0 0	87.1		18 1		7.55		..	..
		19 0	76 2	52 5	14 3	59 9	7 45	7 30	90	10
		47 05	82 2	48 0			7 43	7.43	66	34
	2 0 reinjected	67·45	102 0	89 1			7.43	7.28	82	18
		95.50	81.2	12 6	10 3		7 43	6 5	Relatively acellular	
		114.40	86 1	9 9	11.3	147 8	7 43	6 5	8 (degenerated)	92
8–1*	3 0	0 0	108 9		9 2		7.33		..	..
		20 0	94.1	49 5	13 8	39 6	7 40	7 30	Acellular	
		46 15	141.1	76 8	14 4	48 9		7.17	84	16
		68:15	108 9	42 6	27.4	69.7	7.45	7.33	90	10
	1 5 reinjected	95.45	141.6	46 0	24 5	58 0	7 48	7.33	88	12
		115 0	81 2	12 9	10 0	110 5	7 48	6 83	Relatively acellular	
		140 0						6 6	4	96

Gerhardt's Test

To 5 ml. of urine is added, drop by drop, a solution of ferric chloride (3 per cent.). White ferric phosphate is precipitated. More ferric chloride is added and the solution is filtered. A brown or purplish colour is obtained depending on the amount of acetoacetic acid present. The urine tested must be free from salicylic acid and salicylates and the compounds excreted after administration of aspirin, antipyrin, etc., all of which give a very similar reaction to acetoacetic acid, but never give the sodium nitroprusside reaction.

This test detects 1 part of acetoacetic acid in 7,000 parts of urine. The colour, if due to acetoacetic, is discharged on heating; if due to salicylates, it persists.

Notes.—When Rothera's test is found positive, Gerhardt's test should always be done in order that a rough estimate of the degree of ketosis may be made. When Rothera's test is negative no acetone bodies are present and other tests become unnecessary.

The nitroprussic reaction is a test for acetone, but acetoacetic acid decomposes so readily into acetone that it also gives the test. The ferric chloride test for acetoacetic acid is not given by acetone.

SPECIFIC GRAVITY OF URINE

The urinometer is placed in the urine in the urinometer cylinder or a 50 ml. graduate. The urinometer should float freely in the urine and not touch the side of the vessel. In reading the urinometer the eyes should be on a level with the top of the meniscus of the urine both at the front and back of the cylinder. On the stem of the urinometer the number just visible above the surface of the urine is noted. This is the specific gravity to which 1000 is to be added.

ALBUMIN

Protein (in significant amount) in urine may be albumin and globulin (derived from the blood plasma) or "Bence-Jones protein."

drops rapidly while the concentration of lactic acid correspondingly increases (Fig. 22). The hydrogen ion concentration increases to a pH of 6.5. Polymorphonuclear leukocytes are found wanting or appear in small numbers as degenerated, swollen, or distorted cells. The bulk of the cellular ele-

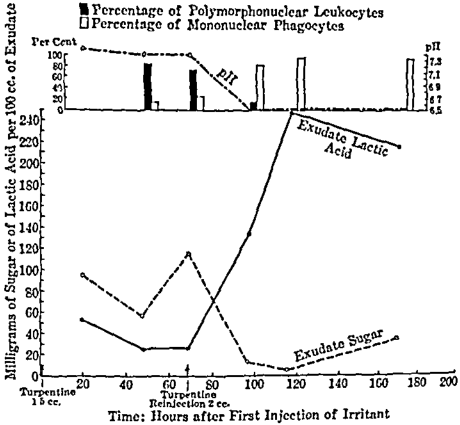


FIG. 2?. ...
sion of
(Dog 9-
rise in ...
acidosis is accompanied by a shift in cellular elements. The polymorphonuclear leukocytes are replaced by mononuclear macrophages. (From Menk... and Warner, *Am. J. Path.*, 1937, *13*, 25)

ment consists of mononuclear phagocytes (cf. Figs. 22 and 23). Frequently on the 1st day following the injection of the irritant, the exudate is found to be relatively acellular. Comparison of lactic acid and sugar figures in both blood and exudate reveals an interesting type of reciprocal relationship

Quantitative Estimation of Albumin

The urine and sulphosalicylic acid are mixed in the proportions named above in a small tube of standard size. After 5 minutes, the turbidity is compared with that in the set of gelatin permanent standards (King and Haslewood 1936; see page 151), and read as mg. of albumin per 100 ml. of urine. If the amount is greater than 100 mg. albumin per 100 ml. the urine is suitably diluted and a fresh estimation carried out.

NOTE.—The above tests will detect albumin in urine in amounts as little as 10 mg. per 100 ml. Unlike the nitric acid tests they do not give "false positive" results.

BLOOD

Using boiled and cooled urine the benzidine or guaiacum test is carried out, as described on page 75. Urine containing blood frequently gives a positive test for albumin.

UREA

Estimations of the amount of urea and of ammonia in the urine are valuable in a number of tests of renal function. The amount of urea in a single specimen may be from 0·02–4·00 g. per 100 ml., while the quantity of ammonia is usually small. The 24-hourly excretion is, approximately, 30 g. of urea and 0·8 g. of ammonia. In certain conditions the ammonia content of the urine may be greatly raised (e.g., in diabetic acidosis).

PRINCIPLES OF METHODS

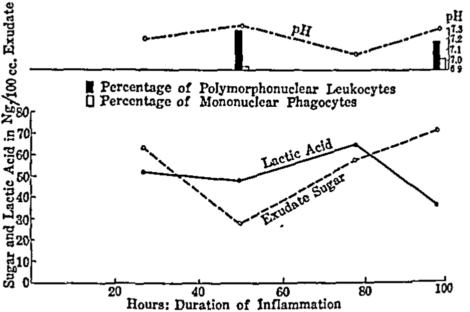
(*a*) The urea is converted to ammonia with urease and the ammonia nitrogen estimated by Nesslerization.

(*b*) The urea is converted to nitrogen by sodium hypobromite and the nitrogen measured manometrically.

Method (*a*) is used for the accurate estimation of urea and ammonia, while method (*b*) is particularly well adapted for use in renal function tests where a rapid and fairly accurate measure of the amount of urea + ammonia is required.

The studies of Lundsgaard (1931) indicated that a muscle poisoned with iodoacetic acid eliminates the production of lactic acid during the contractile phase. The introduction of iodoacetic acid or of 0.001M solution of mono-iodo-acetamide into the inflamed pleural cavity of dogs, however, failed to inhibit the glycolytic activity.

The foregoing observations indicate that the cytological picture in an area of acute inflammation is apparently con-



[illegible] the exudate [illegible] an alkaline range and the polymorphonuclear cells predominate throughout the course of the inflammatory reaction. (From Menkin and Warner, *Am J. Path.*, 1937, *13*, 25)

ditioned by the local pH, which in turn is determined by the rate of lactic acid formation and the depletion of the alkali reserve at the site of injury. With progress in the intensity of the inflammatory reaction there is a tendency for increased glycolytic activity as revealed by a rise in lactic acid formation. This is accompanied by a fall in the carbon dioxide capacity and the pH correspondingly drops. A *true* lactic acid acidosis results at the site of inflammation. The poly-

To express the results as urea multiply by 2·14.

B. Ammonia. *One ml. of the urine is placed in a 50 ml.* volumetric flask together with about 25 ml. of water; 2 ml. of sodium hydroxide and 2 ml. of zinc sulphate are added and, after diluting to the mark, the contents are filtered. Ten ml. of the filtrate (≡ 0·2 ml. of urine) are pipetted into another 50 ml. flask, water is added to about 40 ml. and after the addition of 5 ml. of Nessler's reagent and dilution to the mark, the colour is read in a colorimeter in the same way and using the same standards as described for urine urea + ammonia.

CALCULATION

(1) When the standard is 10 ml. of ammonium chloride,

$$\text{Urine ammonia}^* \left\{ = \frac{\text{Reading of standard}}{\text{Reading of test}} \times 0{\cdot}1 \times \frac{100}{0{\cdot}2} \right.$$

(2) When the standard is 20 ml. of ammonium chloride,

$$\text{Urine ammonia}^* \left\{ = \frac{\text{Reading of standard}}{\text{Reading of test}} \times 0{\cdot}2 \times \frac{100}{0{\cdot}2} \right.$$

* mg. N per 100 ml.

NOTES.—If urea only is required this is of course calculated by subtracting the ammonia value from urea + ammonia figure.

For the purpose of the urea clearance test the urea + ammonia figure is used since, as Van Slyke has pointed out, this result is a better index of the rate of urea excretion.

Hypobromite Method

The apparatus sketched is used. The 50 ml. burette A is clamped vertically and its top end is closed with a doubly bored rubber bung. Through one of the holes in this bung passes a short glass tube closed by a piece of pressure tubing and a clip B. *Through the other hole passes a short tube* connected with pressure tubing to the small bottle C, which is closed by a rubber bung. The other end of the burette is connected to the reservoir D, which is filled with water.

function of the local hydrogen ion concentration. The macrophages are more resistant than the polymorphonuclear cells to changes in hydrogen ion concentration. These *in vitro* observations indicate the primary and direct effect of the pH in determining the cellular constituents of inflammation.[1] The replacement of polymorphonuclear cells by

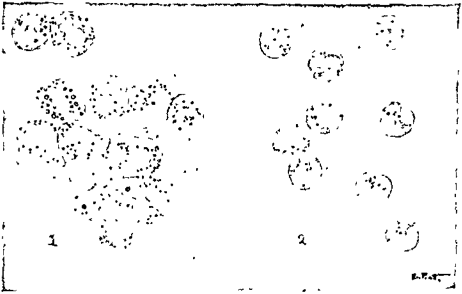


Fig. 26. Effect of pH on leukocytes. 1, a group of supravitally stained

posed to the acid reaction there is now no sign of cellular aggregation; the staining of the cytoplasmic granules is normal, and there is no dye in the nuclei. (From Menkin, *Arch. Path.*, 1939, 27, 115.)

macrophages in the course of inflammation is reasonably explained on the basis of a developing local acidosis in the inflamed area. With further increase in the local hydrogen ion concentration, both types of leukocytes are actively affected and a state of suppuration follows. It is also possible that the development of an acid pH favors the action of pro-

1 As pointed out previously diapedesis of polymorphonuclear leukocytes is apparently an independent phenomenon which is referable to the liberation of leukotaxine

Hence, 60 g. urea ≡ 22·4 l. of nitrogen at N.T.P.
∴ 1 g. „ ≡ 373 ml. „ „ „ „

In fact, 357 ml. of nitrogen are yielded on the average by 1 g. of urea, and it is not necessary to correct for temperature and pressure.

Hence, $357 \text{ ml. } N_2 = 1 \text{ g. Urea}$

$$\therefore \quad \text{Urea in urine}^* = \frac{\text{ml. } N_2}{357} \times \frac{100}{2}$$

$$= \frac{\text{ml. } N_2}{7 \cdot 14} = \text{ml. } N_2 \times 0 \cdot 14$$

* g. per 100 ml.

Reagent.—Sodium hypobromite (prepared fresh, daily). 2·5 ml. of bromine are carefully added to 25 ml. of sodium hydroxide (40 g. per 100 ml.).

TOTAL NITROGEN OF URINE

Total nitrogen of urine consists of the nitrogen-containing compounds: ammonium salts, urea, uric acid, creatinine and small amounts of other substances, some of them still unidentified. The total nitrogen excreted by a normal adult individual is about 14 g. per day. This amount may be depressed in conditions involving kidney damage.

PRINCIPLE

The Kjeldahl Method

The organic substances of the urine are destroyed by digestion with concentrated sulphuric acid and all the nitrogen present is converted into ammonium sulphate. By the addition of excess of alkali, ammonia is liberated and is quantitatively distilled off into a measured amount of standard acid. The amount of acid neutralized by the ammonia gives the measure of the amount of nitrogen present in the sample of urine used.

intermediary product in the breakdown of glycogen to lactic acid. Cori and Cori (1934) have demonstrated that tetanic stimulation of muscle yields hexosephosphate. Under anaerobic conditions the removal of this compound is affected by glycolysis for it is accompanied by an equivalent increase in lactic acid and by the liberation of inorganic phosphate. Menkin and Warner (1937) have accordingly studied the inorganic phosphate content of cell-free exudates and compared it with that in samples of blood serum. In the initial stages of inflammation the level of phosphates in exudates is slightly higher than in serum. After several days, however, when the inflammatory reaction has progressed in intensity and the exudate assumes a purulent character, the phosphate content is found to be several times more elevated in the cell-free exudate than in the serum. Concomitantly, as pointed out above, the lactic acid concentration is found to be considerably augmented. This would strongly suggest a similarity between the changes in carbohydrate metabolism during muscular activity and those occurring in an acutely inflamed area where there presumably develops a state of relative anoxemia owing to impaired local vascular and lymphatic circulation (Menkin, 1931).

Rubel (1936) has recently studied the relation between glycolysis and proteolysis in tissues. The observations of this investigator indicate that an increase in glycolytic activity of tissue is accompanied by an accumulation of amino and non-protein nitrogen, whereas inhibition of glycolytic activity is followed by a diminution of proteolytic processes. It may be noted here that parallel determinations in exudate and blood serum have invariably revealed a higher concentration of amino acid nitrogen in the exudate than in the blood serum (Menkin, 1936*b*, 1938). This difference in level was particularly striking in the later stages of the inflammatory reaction at a time when the lactic acid concentration was elevated. Furthermore, the concentration of total protein nitrogen was found to be lower in the exudate than in the corresponding samples of blood serum (Tables II and III,

tube dips below the surface of the liquid. Ten ml. of concentrated alkali (40 per cent. $NaOH$) are now added to the distillation apparatus through the small funnel. The ammonia which is liberated is distilled over into the standard acid in the conical flask by bubbling steam through the mixture. A Bunsen burner is placed under the large round bottomed boiling flask which should be about half full of water. Steam is bubbled through the mixture and the distillation continued until the contents of the conical flask are about twice what they originally were. The flask is now lowered until the end of the condenser tube no longer dips into the liquid. The condenser tube is washed down with distilled water into the conical flask, which is now removed and its contents titrated with 0·1 N-sodium hydroxide. *The titration figure so obtained is subtracted from the 10 ml.* of 0·1 N-sulphuric acid *to give the* ml. of standard acid which were neutralized by the ammonia.

CALCULATION

ml. 0·1 N-H_2SO_4 neutralized by ammonia × 1·4
= mg. nitrogen in the 1 ml. of urine.

It is usual to express the nitrogen content of urine in terms of the output per 24 hours.

NOTE.—Determinations of the different nitrogen constituents of urine are made according to procedures which are essentially the same as those used for blood.

CREATININE AND CREATINE

Creatine is methyl-guanidine-acetic acid.

Creatinine is its anhydride. These substances are probably derived from protein.

The excretion of *creatinine* in the normal individual varies according to the muscular weight. The amount eliminated each 24 hours ranges between 0·4 and 1·8 g. in the adult. *On a creatine-free diet the output is remarkably constant and* very seldom varies except in uncommon clinical conditions.

acidosis in acutely inflamed areas. But their contention seems to be invalidated by a dubious technique utilized in measuring the hydrogen ion concentration, and by their failure to correlate the leukocytic picture and pH on an identical sample of exudate (for detailed criticisms see Menkin, 1939*b*). The writer's conclusions on this question have been recently further confirmed by Lurie (1937). Finally, it is conceivable that the relative absence of leukocytic migration in the late stages of inflammation may be referable to a local increased concentration of lactic acid. This acid is, as has been pointed out in the preceding chapter, negatively chemotactic. Its formation in appreciable amounts may thus counteract the migration of white cells induced by the liberation of leukotaxine.

Summary. The usual cellular sequence in the development of an acute inflammatory reaction consists of an initial infiltration of polymorphonuclear leukocytes which is subsequently replaced by an abundance of macrophages. The underlying mechanism involved in this basic phenomenon depends on the production of a local acidosis in the inflamed area.

At a pH varying between 7.2 and about 7.4 the predominating cell of an exudate is the polymorphonuclear leukocyte. Between 7.0 and 6.8 the macrophage constitutes the chief cellular element. Concomitant with the rise in hydrogen ion concentration, the polymorphonuclear leukocyte manifests various signs of degeneration, injury, or necrosis. Below a pH of 6.7 or thereabouts, all types of cells are injured and a state of frank suppuration ensues.

The correlation between the pH and the cytological picture in inflammation suggests that the hydrogen ion concentration is the factor conditioning the cellular pattern of an exudate. *In vitro* studies add further support to the view that the viability of leukocytes in an inflamed area is a direct function of the pH. The hydrogen ion concentration thus determines the survival of leukocytes; but the diapedesis of polymorphonuclear leukocytes into inflamed tissue is refer-

urine (usually equal to the volume used for the creatinine determination), and about 130 ml. water. The contents are brought to the boil rapidly, and then allowed to boil gently for 1 hour. The flame is raised and the mixture boiled down rapidly to bring the volume of the solution to about 25–30 ml. The flask is cooled, and 4 ml. N-NaOH are added. After 10 minutes the contents of the flask are transferred to a 100 ml. volumetric flask and water is added to the mark. The contents are compared in the colorimeter with the standard coloured solution used for creatinine.

CALCULATION

As for creatinine. The result is total creatinine, i.e., preformed creatinine plus creatine, expressed as creatinine. Thus, the amount of preformed subtracted from the total creatinine gives the creatine content of urine expressed as creatinine.

SOLUTIONS

N-*Sodium Hydroxide.*

Saturated Solution of Picric Acid.

Creatinine "Stock" Standard. (Containing 1 mg. of creatinine per ml.).—1·602 g. of pure creatinine zinc chloride are dissolved in N/10 hydrochloric acid solution, and the volume made up with the N/10 acid to 1 litre. (This is the same "*Stock*" *Standard* as is used in the blood method.)

URIC ACID

Urine contains about 0·4 g. of uric acid per 24-hourly specimen. This amount may be increased in cases of leukaemia, and decreased in gout.

PRINCIPLE

Urine is diluted and treated with Benedict's arsenophosphotungstic acid reagent and then with a sodium cyanide-urea solution. The blue colour produced by reduction, at the

CHAPTER VII

THE PHAGOCYTIC THEORY

Metchnikoff may well be considered the founder of the phagocytic theory. To this investigator phagocytic activity is the basis of inflammation. It represents a reaction by the host against irritants, and as such it is regarded by Metchnikoff as an adaptation in the evolutionary development accompanying the struggle for existence (1892).

Metchnikoff undertook a comparative study of phagocytosis in the various zoological phyla from protozoa to mammals. He was able to show that in more primitive forms phagocytosis was essentially a nutritional function. After the ingestion of food by an amoeba, intracellular digestive enzymes disposed of the particle, or else it was extruded. The significance of his work on invertebrate forms was confirmed and further extended by Messing (1903) and by more recent work on earthworms by Cameron (1932).

In the higher forms phagocytosis assumes a more specialized function. Two types of leukocytes are primarily engaged in phagocytosis: the polymorphonuclears and the macrophages. Metchnikoff first established the importance of these cells in inflammation and immunity in view of their phagocytic ability. It is true, however, as pointed out by Lubarsch (1925), that under certain circumstances many other types of cells are capable of manifesting phagocytic function. Metchnikoff sharply differentiated the ingestive ability of both types of leukocytes. Macrophages presumably were concerned with the phagocytosis of tissue cells, whereas microphages or polymorphonuclear leukocytes tended to ingest bacteria. Chronic infections, such as tuberculosis or leprosy, were regarded as exceptions to the rule. The available evidences indicate that this earlier view is probably not strictly

CHLORIDES

The usual excretion of chloride amounts to about 10 or 12 g. (expressed as sodium chloride) per 24 hours for normal individuals. The amount of this excretion may be altered during certain clinical conditions. It is most markedly depressed in conditions of oedema which are characterized by salt and water retention.

PRINCIPLE

The Volhard procedure for the determination of chloride in solution consists in the precipitation of the chloride with a measured excess of standard silver nitrate in the presence of nitric acid. The excess of silver nitrate over that necessary for complete precipitation is measured by titration with standard ammonium thiocyanate. A small amount of a ferric salt is added as indicator, the first production of a red colour due to the formation of ferric thiocyanate indicating the point at which an excess of ammonium thiocyanate has been added. The ferric salt is included in the silver nitrate solution.

The difference between the volume of the standard silver nitrate used and that of the standard ammonium thiocyanate represents the amount of silver precipitated by the chloride.

METHOD

Ten ml. of urine are added to about 25 ml. of water in a 100 ml. volumetric flask. Twenty ml. of the standard silver nitrate are added and after a few minutes the volume made to the 100 ml. mark. The mixture is filtered, and 50 ml. of the clear liquid transferred to a 250 ml. conical flask for titration. Standard ammonium thiocyanate is run in until the production of the first permanent faint reddish-pink colour.

CALCULATION

The figure representing the thiocyanate titration is subtracted from 10 (ml. of silver nitrate) to give the figure representing the silver nitrate precipitated by chloride.

2. The early work of Ledingham (1908) suggested very definitely that the rate of phagocytosis increased with a rise in temperatures between 18° C. and 37° C. Madsen and Wulff observed that the temperature for maximum phagocytosis seemed to coincide with the body temperature of the given species (1919). For instance, chickens and pigeons showed maximal phagocytosis *in vitro* at 41° C. whereas human leukocytes displayed an optimum reaction at 37° C. Fenn critically studied the experimental observations of Madsen and Watabiki (1919) and arrived at the conclusion that Q_{10}, the temperature coefficient, was 2.0 indicating thus that the rate of phagocytosis was doubled by a rise of 10 degrees in temperature. When Fenn analyzed his own material he stated, however, that the values of Q_{10} may vary at different temperature levels. This, he thought, might possibly be referable to the introduction of additional factors with temperature changes, such as cellular viscosity, clumping of cells or of particles.

3. In regard to the effect of osmotic tension, the work of Hamburger (1912) indicated that hypotonicity as well as hypertonicity tend to inhibit phagocytosis of carbon particles by the leukocytes of the horse. To some extent the effect was found to be reversible, especially in the case where cells had been first subjected to a hypotonic medium. Placing them back into an isotonic solution induced a normal phagocytic reaction. The studies of Wright and Reid (1906), and those of Ouweleen (1917) have cast some doubt, however, on Hamburger's conclusion regarding the depressing effect of hypotonicity. These investigators found maximal phagocytosis to occur in 0.6 or 0.5 per cent NaCl.

4. A number of investigators have studied the rôle of ions in phagocytosis. Hamburger (1912, 1927) described the depressing effect of I ions; some of the other halogens, on the other hand, produced relatively little difference. The effect of some ions is reversible. By placing the cells from an iodized medium to one of NaCl, the normal rate of phagocytosis may be restored. Ca ions have the peculiar property of

acetate method for sodium in the same manner as blood plasma.

POTASSIUM

The cobaltinitrite method for blood serum is applied to a sample of the urine from which the ammonia, which forms an insoluble cobaltinitrite like potassium, has been eliminated by ashing and re-solution in dilute acid.

Two ml. of urine are evaporated to dryness in a good quality porcelain, vitreosil or platinum crucible. The residue is ashed at a dull red temperature (about 400° C.) for some hours. Overnight is convenient if an electric muffle is available; otherwise the crucible may be placed inside two porcelain evaporating dishes, one inverted over the other, and heated with a Bunsen burner until all carbonaceous matter has disappeared.

The ash is dissolved in a small amount of 0·1 N-hydrochloric acid, and the solution transferred quantitatively with washings of water to a 10 ml. standard flask, made to volume and mixed. (If the potassium content is likely to be high, the solution of the ash is made to 15 or 20 ml.)

0·5 ml. of this solution (= 0·1 ml. of urine) is used for the potassium determination described for blood serum. The result so obtained is multiplied by 5 to allow for the dilution of 2 ml. of urine to 10 ml.

BILE PIGMENT

Urine containing bile is generally yellowish-green to brown in colour. When shaken it foams readily because of the presence of the bile salts. The foam has a yellowish-green colour. This in itself is a rough test for bile but the more accurate clinical tests depend upon the fact that bilirubin is oxidized by nitric acid to form a series of coloured compounds, biliverdin (green), bilicyanin (blue), choletelin (yellow), etc.

Nitric Acid Test

When a urine containing bile pigment is filtered the bilirubin is largely retained on the filter paper. Twenty to

b. Histamine augments phagocytosis in a concentration of about 1:40,000.

c. Extirpation of the thyroid in the rabbit decreases phagocytosis (*Asher, 1924; Furuya, 1924; Masuno, 1924; Abe, 1925*). Furuya succeeded in increasing phagocytosis in a thyroidectomized rabbit by feeding thyroid extract.

d. Bacteriophage, according to D'Herelle (1926) seems to be adsorbed on susceptible bacteria, rendering them thereby a more favorable target for phagocytosis. These observations, according to Mudd, McCutcheon, and Lucké (1934), suggest the necessity of studying the interfacial relationship between leukocytes and bacteria containing adsorbed bacteriophage.

Mechanism of Phagocytosis

The important phagocytosis-promoting substances in normal and immune sera are the *opsonins* and *tropins*. These substances, by forming a film around the particles to be phagocytosed, favor their ingestion. The experimental studies of Lucké, McCutcheon, Strumia, and Mudd (1929, 1934) have led them to the following interpretation of the basic mechanisms involved: Serum proteins are deposited on the surface of the particles. This, by altering the relationships in the interface between particle and phagocyte, and between particle and medium, favors the spreading of the *leukocyte over the surface of the object to be phagocytosed.* The experimental procedure adopted by these investigators consisted in correlating surface properties and phagocytosis of bacteria, red blood corpuscles, and collodion particles in serial dilutions of normal or immune serum. The surface properties were studied in regard to a) agglutination, b) cohesiveness by means of a modified agglutination or "resuspension" reaction, c) cataphoresis, and d) wetting properties of acid-fast bacteria and erythrocytes in an oil-water interface before and after immunization. The changes in these surface properties with serial dilutions were correlated with alterations in phagocytic ability. The correlation between

2. 0·5 ml. of glacial acetic acid, followed by 2 ml. of 5 per cent. copper sulphate, are added to 10 ml. of urine. Two ml. of chloroform are added and the mixture thoroughly shaken and allowed to settle. If any urobilin is present it will appear in the chloroform layer, to which it imparts a pink colour. The spectrum may again be examined for absorption at the green-blue.

INDICAN

Only small amounts of indican, 5–20 mg. per day, appear in normal urine. Great increases are found in clinical conditions where there is stagnation and putrefaction of intestinal contents. Likewise decomposition of body proteins due to bacterial action, as in gangrene and putrid pus formation, results in large amounts of indican appearing in the urine.

TEST

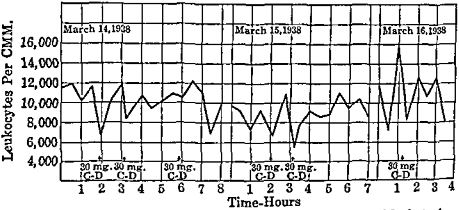
To 5 ml. of urine in a test-tube are added 5 ml. of concentrated hydrochloric acid and 2–3 drops of *dilute* sodium hypochlorite solution, or of a freshly made saturated solution of bleaching-powder (a little bleaching-powder shaken in 10 ml. of water in a test-tube at room temperature). 2–3 ml. of chloroform are added and the test-tube closed with the thumb and inverted several times. The amount of indican in a normal urine will give a light blue colour in the chloroform in this test: if indican is present in increased amount, the chloroform will be intensely coloured. The test is therefore "controlled" with a normal urine, tested at the same time.

Care must be taken in the above test not to use a large excess of hypochlorite solution, or the blue-coloured chloroform-soluble product will be oxidized to a colourless substance.

MELANIN

The black melanin pigments do not occur in normal urine. Their occurrence is usually associated with melanotic tumours.

The effect of leukotaxine on the number of leukocytes in the circulating blood. Experiments were performed on rabbits. When, after several preliminary counts on peripheral blood samples, the normal leukocytic rhythm had been established, leukotaxine, extracted as previously described from inflammatory exudates of dogs (Chapter IV), was injected into rabbits, either subcutaneously or intravenously. The results of numerous experiments show that repeated injections of this substance even over a prolonged interval of time (Fig. 27) likewise fail to increase the level of leukocytes in the circulating blood stream. The conspicuous degree of cellular infiltration and abscess formation occasionally en-



...of leukotaxine ...ays, to induce ...ations.

countered at the site of repeated leukotaxine injections bears evidently no direct relation to the number of circulating leukocytes.

Presence of a leukocytosis-promoting factor in inflammatory exudates. Having apparently eliminated leukotaxine as the factor responsible for the state of leukocytosis frequently associated with inflammation, studies were undertaken by the writer in an endeavor to determine whether the active principle might not be liberated in the exudate as a result of tissue injury.

Danzer (1930) showed that *the injection in rabbits of* various extracts of organs deflects the polynuclear count.

test-tubes are then placed in a rack and 4 ml. of the buffered urine (of dilution 1 : 5) are placed in tube (1). Two ml. of buffer are added to each of tubes (2) to (7). Two ml. from tube (1) are mixed with the contents of tube (2). Two ml. of this mixture from tube (2) are transferred to tube (3), and so forth until tube (7) is reached, from the final contents of which 2 ml. are discarded. The dilutions are then 1 : 5 in tube (1), 1 : 10 in tube (2), 1 : 20 in tube (3), and so on, to 1 : 320 in tube (7).

One ml. of 0·2 per cent. starch solution is then added to each tube and mixed with the other liquids. The tubes are incubated, by immersion in a water-bath, at 38° C. for 30 minutes. At the end of this time 3 drops of N/50 iodine are added to each tube.

CALCULATION

Let the dilution of the urine in the first tube which shows no blue or mauve colour be $1 : x$

This tube contains :

2 ml. of a $1 : x$ dilution of urine, i.e., $\frac{2}{x}$ ml. of urine.

Therefore $\frac{2}{x}$ ml. of urine contains just sufficient diastase to digest 1 ml. of 0·2 per cent. starch solution ; that is,

$\frac{2}{x}$ ml. of urine digests 2 ml. of 0·1 per cent. starch solution;

hence,

1 ml. of urine digests $\frac{2}{2/x} = x$ ml. of 0·1 per cent. starch soln.

Therefore, the number of Wohlgemuth units is equal to x, the dilution factor of the tube in which the digestion of the starch is just complete.

Urinary diastase is usually reported in terms of the output of the enzyme for 24 hours.

TABLE VIII

EFFECT OF INFLAMMATORY EXUDATES INJECTED INTO THE CIRCULATION ON LEUKOCYTE COUNTS

Dog No.	Absolute Number of Leukocytes before Injection of Exudate	Highest Level in the Absolute Number of Leukocytes within Six Hours after Injection of Exudate	Per Cent Increase in Leukocytes
	*per mm*³	*per mm*³	
4-14	10,525	24,275	130 6
4-27	9,850	20,050	103 5
4-17	7,200	14,000	94.4
4-14	9,975	18,800	88 5
4-17	7,875	14,625	85 7
4-27	14,650	25,550	74 4
4-27	14,150	25,600	71.2
4-14	9,175	14,600	59.1
4-23	8,750	13,725	57.4
4-19	12,100	18,725	54.8
4-14	10,900	16,600	52.3
4-14	8,850	13,100	48.0
4-26	9,050	12,900	42 5
4-14 *	11,425	14,375	25 8
Average	10,377	17,638	70 0

* Exudate obtained from dog having a profound leukopenia (W.B C = 3,125) resulting from two successive injections of turpentine

cubic millimeter. These and other similar experiments suggest that an exudate from an animal with marked leukopenia contains a reduced amount of leukocytosis-promoting factor.

Several hundred cubic centimeters of exudate can often be recovered from the pleural cavity of a dog after a single injection of 1.5 cc. of turpentine. Yet a sample of 15 to 25 cc. of this exudative material introduced into the blood stream *of a normal animal induces, as shown above,* a definite increase in the number of circulating leukocytes. The bulk of recovered exudate would doubtless considerably dilute the irritant. For this reason, it seems somewhat doubtful that the leukocytosis-promoting effect of exudates can be referred to the direct action of turpentine or some of its derivatives.

In an endeavor, however, to throw further light on this point, experiments were devised in which turpentine mixed in serum, or the exudative material recovered after physical

PRINCIPLE

Ascorbic acid rapidly reduces, in acid solution, the dye 2 : 6-dichlorophenol-indophenol to a colourless substance. Urines containing ascorbic acid also effect this reduction and the amount of dye reduced by the urine is a fairly accurate measure of the quantity of the acid present.

METHOD

An appropriate amount of the dye (usually 0·5 ml. of :he solution of 2 : 6-dichlorophenol-indophenol) is measured nto a small test tube. A drop of 50 per cent. acetic acid ; added and the urine rapidly run in from a 2 ml. burette until the red colour of the dye disappears.

CALCULATION

0·5 ml. of 2 : 6-dichlorophenol-indophenol solution contains 0·04 mg. of dye. This amount is reduced by approximately 0·02 mg. ascorbic acid. For accurate work, the dye solution should be checked against the pure vitamin and should be freshly prepared daily.

The amount of ascorbic acid equivalent to the dye taken is contained in the quantity of urine used to decolourize the dye, whence the amount present in the total sample or in 100 ml. of urine is calculated. Where acetic acid has been added as preservative the volume added must be allowed for in the calculation. Results are expressed in mg. ascorbic acid per 100 ml. of urine or as mg. excreted in 24 hours, according to the information desired and the type of test being conducted.

Example. A sample of urine had a total volume of 720 ml. including 100 ml. of glacial acetic acid.

0·5 ml. of dye solution required 0·82 ml. of the acidified urine.

∴ 0·82 ml. of the acidified urine contained 0·02 mg. of ascorbic acid

Hence 720 ml. of the acidified urine contained $\left.\right\} \dfrac{0 \cdot 02}{0 \cdot 82} \times 720$

$= 17 \cdot 6$ mg.

20 cc. of exudate (Table VIII, cf. Dogs 4-14 and 4-27). In an experiment of this sort, the stimulus on the hematopoietic centers is probably of short duration. On the other hand, with an acute inflammatory reaction in the pleural cavity, there is presumably a continuous supply of the leukocytosis-promoting factor diffusing into the systemic circulation.

To determine whether neurogenic influence is effective in the production of leukocytosis with injury, Doctor Luco of the Department of Physiology denervated the leg of a dog by cutting the sciatic, the femoral, the obdurator, the femoro-cutaneous lateralis and posterior nerves. The abdominal sympathetic strands were likewise extirpated. The dog was subsequently subjected, under nembutal anesthesia, to a severe burn, by dipping its denervated limb for one minute into water heated at 90° to 95° C. Extensive edema developed rapidly. On the following day the white blood cell count revealed marked leukocytosis (Table IX). This experiment probably points to the absence of any significant nervous mechanism in the regulation of leukocytosis with concomitant inflammation.

In conclusion, the results summarized in Tables VIII and IX clearly show that the products of injury liberated in an area of acute inflammation are *per se* capable of inducing in a normal dog a prompt leukocytosis to a degree reasonably comparable with that seen in an animal with an induced pleurisy.

The effect on the leukocyte level obtained after the administration of exudative material transcends the maximum rise occurring during the rhythmical leukocytic fluctuations seen in a dog. Such an animal reveals, as a rule, relatively little variation from hour to hour. During the period of experimentation, i.e. six to eight hours, the maximum increase in leukocytes of several normal dogs averaged 23.8 per cent (Table X). The counts were made on dogs which, on separate days, had been injected with exudates (cf. Table VIII). A comparison of Tables VIII and X indicates that there is therefore roughly, as a result of a single intravascular injec-

following details if reliable results are to be obtained. Four cylinders are marked: A and B (blanks), T (test) and S (standard), and the following amounts of urine, etc., placed in them.

	A	B	T	Standard
Extracted urine	2 ml.	2 ml.	2 ml.	1 ml. of dilute standard.
Methanol	2 ml.	2 ml.	2 ml.	0·5 ml. water, 2 ml. of methanol.
20 % sodium hydroxide	1 ml.	1 ml.	—	—

To cylinder A is added 2 per cent. potassium ferricyanide drop by drop from a burette until the colour remains slightly more yellow than cylinder B for 30 seconds. The volume added is noted, and this tube is discarded. The remaining tubes are treated as follows:

	B	T	Standard
2 % potassium ferricyanide	—	Same amount as used in tube A.	1 drop.
20 % sodium hydroxide	—	1 ml. (mix).	1 ml. (mix).
Hydrogen peroxide 5 % (20 vol.)	5 drops.	5 drops.	5 drops.
Iso-butanol	10 ml.	10 ml.	10 ml.

All three cylinders are shaken vigorously for 2 minutes and the mixtures allowed to separate; the aqueous lower layers are removed and discarded. Four ml. distilled water are added to each cylinder and shaken for 2 minutes, allowed to separate, the water removed and 2 ml. of ethanol added to clarify. The volumes of B, T and Standard are made up to 15 ml. with redistilled *iso*-butanol, and 10 ml. of B and T are used for matching fluorescence.

Fluorimetry. Two tubes are labelled B and T. Ten ml. of the *iso*-butanol extract B are measured into tube B, and a similar quantity of T into tube T. The comparison is made in a completely dark room using an ultra-violet lamp, which is enclosed in a case fitted with a Woods glass filter. The tubes are held in front of the lamp and the fluorescence viewed down the long axis of the tubes. The bottoms of the tubes rest on a blackened platform fitted to the case of the lamp. Precautions are taken to interchange the position of the tubes and to inspect the fluorescence quickly to avoid errors due to fatigue. Standard thiochrome (S) is added to tube B drop by drop with mixing, and at the same time

circulating blood of normal animals and of dogs previously treated with exudates. A leukocytosis invariably failed to develop in all these experiments. The administration of dead bacteria (broth culture of killed Staphylococcus aureus) likewise proved incapable of altering significantly the total number of leukocytes.

Type experiments are graphically represented in Figures

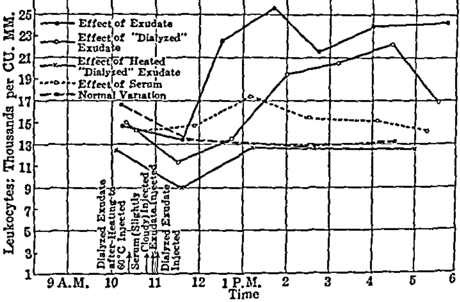


Fig. 29. A comparison of the effect of a sample of exudate on the blood leukocyte level of Dog 4–27 with that obtained with the residual dialyzed fraction of a sample taken from the same exudate. Note that the material retained in the cellophane bag after prolonged dialysis has, to a considerable degree, retained the leukocytosis-promoting factor. Heating to 60° C. this residual dialyzed fraction completely inactivates the factor (dash and dotted line). The effect of blood serum and the normal variations in the counts of this animal are likewise indicated.

28 and 29. When the exudate is reinjected in a dog several hours following a first injection, the primary effect can be reinforced and a secondary rise in the level of leukocytes may become manifest (Fig. 28). The transitory leukopenia immediately following the intravascular injection of serum (Fig. 28) or even of exudate (Fig. 29) recalls the leukopenia described by Ewing (1895) after intravenous injection of

CHAPTER VIII

ANALYSIS OF CALCULI

Calculi, although occasionally composed of a single constituent, are commonly mixtures of substances, and should always be cut or sawn to ascertain the presence or absence of different layers of deposits. The layers should be tested separately.

Renal Calculi. These most often contain carbonate, oxalate, calcium, uric acid or urates, ammonium salts and phosphates; more rarely cystine, "urostealith" (fatty mixtures), fibrin and xanthine.

Biliary Calculi. The following substances may be present in these stones: cholesterol, bile-pigments and calcium.

SCHEME OF TESTS

Renal Calculus

The calculus is powdered in a mortar (separate layers being treated separately). Some of the powder is treated in a test-tube with cold 2 N-nitric acid. Effervescence indicates *carbonate.* The solution is gently boiled, cooled, and filtered. Some of the filtrate is made alkaline with ammonia solution. A precipitate indicates *calcium oxalate* or *phosphate*: the phosphate precipitate being soluble in acetic acid (now added in excess), the oxalate insoluble. The presence of phosphates may be confirmed by addition of ammonium molybdate solution to the nitric acid mixture. A yellow precipitate on boiling, or an intense blue colour on the addition of aminonaphtholsulphonic acid reagent (cf., blood phosphorus), confirms phosphate. The presence of oxalate may be confirmed by its conversion to carbonate by heating some of the precipitate to red heat over a flame and treating the residue with 2 N-hydrochloric acid, when effervescence indicates *oxalate.* This solution is treated

leukopenia following the injection of an exudate, would rule out the rise in the leukocyte level as due to a mechanical transfer of cells contained in the injected exudates.

The leukocytosis-promoting factor of exudates is thermolabile. Boiling the exudate, or merely heating it for several hours at 60° C. inactivates it. This is illustrated in the experiments listed in Table XI. The factor is essentially non-dialyzable. When dialyzed against running tap water through a cellophane membrane for about twenty-four hours, the indiffusible residual material retains a considerable amount of its potency (Table XI). Heating the residual fraction of the dialyzed exudate to 60° C. completely inactivates its potency. The results of these observations are illustrated in Figure 29.

The leukocytosis-promoting factor seems to exert its effect primarily on the hematopoietic tissue in the bone marrow. Following the injection of an exudate, there is a definite rise in the number of immature polymorphonuclear leukocytes. The following data from a type experiment supports this view:

Dog Number 4–27

1. Absolute number of leukocytes prior to injection of exudate	14,650/mm³
(a) Percentage of polymorphonuclear leukocytes	86%
(b) Absolute number of polymorphonuclear leukocytes	12,599/mm³
(c) Percentage of immature polymorphonuclear leukocytes	8%
(d) Percentage of mature polymorphonuclear leukocytes	92%
(e) Absolute number of immature polymorphonuclear leukocytes	1,008/mm³
(f) Absolute number of mature polymorphonuclear leukocytes	11,591/mm³
2. About 20 cc of exudate injected into blood stream	
3 Five hours later· Absolute number of leukocytes	23,825/mm³
(a) Percentage of polymorphonuclear leukocytes	88%
(b) Absolute number of polymorphonuclear leukocytes	20,960/mm³
(c) Percentage of immature polymorphonuclear leukocytes	38%
(d) Percentage of mature polymorphonuclear leukocytes	62%
(e) Absolute number of immature polymorphonuclear leukocytes	7,968/mm³
(f) Absolute number of mature polymorphonuclear leukocytes	12,998/mm³

A survey of the foregoing figures indicates that the absolute number of mature polymorphonuclear leukocytes barely increases after the administration of the exudate. On the other hand, the absolute number of immature polymorphonuclear

hydrochloric acid; 2 N-ammonia; 2 N-sodium and potassium hydroxides; lead acetate (10 g. per 100 ml.); ammonium molybdate (5 g. per 100 ml.); ammonium oxalate (4 g. per 100 ml.); sodium cyanide-urea (5 g. sodium cyanide and 20 g. urea in 100 ml. of solution); Folin's uric acid reagent (see "uric acid" in blood); Erlich's diazo reagent (freshly made mixture of 10 ml. of solution "A" (1 g. sulphanilic acid + 250 ml. N-hydrochloric acid + water to 1 litre) and 0·3 ml. of solution "B" (sodium nitrite, 0·5 g. per 100 ml.)).

SOLUTIONS

2 N-*Nitric Acid.*—12·67 ml. concentrated acid diluted to 100 ml. with water.

2 N-*Ammonia Solution.*—11·4 ml. concentrated ammonia diluted to 100 ml. with water.

2 N-*Acetic Acid.*—12 ml. glacial acetic acid diluted to 100 ml. with water.

Ammonium Molybdate Solution.—5 g. ammonium molybdate dissolved in water and made to 100 ml. (see p. 55).

Aminonaphtholsulphonic Acid.—See p. 55.

2 N-*Hydrochloric Acid.*—20 ml. concentrated acid to 100 ml. with water.

Ammonium Oxalate.—Saturated (4 per cent.) solution (see p. 60).

2 N-*Sodium Hydroxide.*—8 g. per 100 ml.

Folin's Uric Acid Reagent.—See p. 10.

Sodium Cyanide—Urea Solution.—See p. 10.

circulating leukocytes. With high concentrations of the acid, however, in agreement with the earlier studies of Fox and Lynch (1917), there is a preliminary leukopenia accompanied by symptoms of toxicity. This is followed by a beginning rise in the cell count from about the sixth or seventh hour. The cellular increase progresses, producing about twenty-four hours later a state of frank leukocytosis. This retarded development of leukocytosis is unlike the relatively prompt response induced with exudates (cf. Table VIII). It is, however, conceivable that derivatives of nucleic acid, such as adenine or guanine nucleotides, might produce an accelerated leukocytosis. This, as mentioned above, has been shown by Doan and his collaborators to occur when normal rabbits are injected with these compounds. The injection of six milligrams of adenosine (which is essentially a nucleoside of adenine) in saline into the circulating blood of a dog failed to induce prompt rise in the level of leukocytes. The possibility is not precluded, however, that inflammatory exudates may contain considerable quantities of nucleotides which *per se can account for the induced leukocytic response.* Studies are now in progress to test this possibility.[1]

Moon (1938) is of the opinion that histamine or the so-called hypothetical H-substance is of definite significance in the production of leukocytosis associated with inflammatory conditions. This worker finds an increased leukocyte count in cats, following the intravenous injection of one to two milligrams of histamine. The injection of similar or slightly higher amounts (e.g. 5 mg.) in dogs is, in the experience of *the writer, wholly ineffective in altering appreciably the* total leukocyte count. When eight to twelve milligrams of

[1] ... obtained through the courtesy of the Smith, ...

... was 121 9 per cent.

Food debris in the "resting juice," and an abnormal excess of total over free acid indicate pyloric obstruction, with decomposition of the retained gastric contents. The normal excess of "total" over "free" acid is about 10 ml. N/10 per 100 ml.

ANALYSIS OF GASTRIC CONTENTS

Gastric Acidity. Five ml. of the stomach contents are pipetted into a porcelain dish containing 2 to 3 drops of a mixture of equal volumes of Topfer * and phenolphthalein indicators.

The mixture is titrated with N/20 sodium hydroxide until a permanent orange colour is obtained (the point at which all the free acid is neutralized). Titration is then continued until the colour of the solution becomes red (total acid).

The results are multiplied by ten and thus expressed as ml. of N/10 sodium hydroxide required to neutralize 100 ml. of stomach contents.

Bile. A few ml. of the stomach contents are put into a test-tube and a few drops of N/50 iodine added. A green colour denotes bile.

Mucus. Mucus is detected by the stringy appearance of the stomach contents.

Blood. Blood is detected in the same manner as "occult blood" in faeces.

Lactic Acid. 2 ml. of stomach contents in a test-tube are shaken with 5 ml. of ether. The mixture is allowed to separate and the ether layer is removed to another tube.

The ether layer is evaporated off by immersing the tube in hot water.

The residue is dissolved in 2 ml. of water and a few drops of MacLean's reagent are added. A yellow colour indicates the presence of lactic acid.

MacLean's Reagent is a mixture of :—

100 ml. saturated mercuric chloride solution (5 per cent.)
1·5 ml. concentrated hydrochloric acid
5 g. ferric chloride.

* Topfer's indicator is a solution (0·5 g. per 100 ml.) of dimethyl yellow in alcohol.

material. The factor, after prolonged dialysis of the exudate through a cellophane membrane, is found to be, in large part, indiffusible. Recent unpublished studies on protein fractionation of exudates indicate that the active principle is either a pseudoglobulin or that at least it is associated with that protein fraction.[1]

[1] These latest studies will be reported *in extenso* in two forthcoming publications (*Science*, 1940, *Arch. Path.*, July 1940).

Chapter X

HYDROGEN ION CONCENTRATION

In pure distilled water the concentrations of hydrogen ions and of hydroxyl ions are equal. The water is "neutral." In an acid solution there is a preponderance of hydrogen ions over the concentration of hydroxyl ions. In an alkaline solution the concentration of hydroxyl ions is greater than that of hydrogen ions.

The product of the concentrations of hydrogen ions and of hydroxyl ions is the same (i.e., is a constant) in all aqueous solutions, be they acid, neutral or alkaline.

$$(H^+) \times (OH^-) = K_W$$

Pure distilled water has a concentration of one ten millionth gram of hydrogen ions per litre, i.e., $1/10^7$ or more briefly a concentration of 10^{-7} gram per litre. The concentration of hydroxyl ions in distilled water is likewise 10^{-7}. The product of the concentration of hydrogen ion and hydroxyl ion is $10^{-7} \times 10^{-7} = 10^{-14}$, the value of the constant K_W.

Hundredth normal hydrochloric acid (0·01 N-HCl) has a concentration of hydrogen ion of about 0·01 g. per litre, i.e., of 10^{-2}. The hydroxyl ion concentration will, therefore, be 10^{-12}, and the value of the product remains 10^{-14}. Likewise for hundredth normal sodium hydroxide (0·01 N-NaOH) the concentration of hydroxyl ions is 10^{-2} and of hydrogen ions 10^{-12}. And the constant K_W is in both cases 10^{-14}.

The symbol *p*H is used to denote the negative logarithm (to the base 10) of the hydrogen ion concentration, e.g., *p*H 7 for the distilled water of hydrogen ion concentration 10^{-7}, *p*H 2 for the 0·01 N-hydrochloric acid of hydrogen ion concentration 10^{-2}, and *p*H 12 for the 0·01 N-sodium hydroxide whose concentration of hydrogen ions is 10^{-12}. It forms a convenient means for designating the hydrogen ion

vestigators. Muscatello (1895) showed that carmine and various other inert particles, when injected into the peritoneal cavity, reached the anterior mediastinal lymph nodes very rapidly. Noetzel (1906) injected bacteria (*Bacillus pyocyaneus*) into the knee joints of rabbits; from five to ten minutes later he was able to demonstrate the presence of the organisms in the inguinal, crural, and lumbar lymph glands. Buxton (1907) found that typhoid bacilli, within a few minutes after their injection into the peritoneal cavity, appeared in great numbers in the blood stream. Wells and Johnstone (1907) showed that the absorption of bacteria from the peritoneal cavity takes place through lymphatic vessels. New and similar observations concerning normal lymphatic absorption have been reviewed in great detail by Drinker and Field (1933).

Does the inflammatory reaction retard the dissemination of microorganisms from the site of inflammation? The studies of Issayeff (1894) are interesting in this connection. He showed that the peritonitis induced by a variety of sterile irritants, such as foreign blood serum, bouillon, or normal salt solution, temporarily increases resistance to subsequent intraperitoneal injection of bacteria. Cobbett and Melsome (1898) found that a local inflammatory reaction, such as that following the injection of mustard oil, protects the skin of a rabbit's ear against attempts to induce erysipelas. Pawlowsky (1909) repeated the observations of Noetzel and demonstrated the presence of staphylococci in the blood and organs of guinea pigs from twenty-four to forty-eight hours after inoculation of the knee joint. If before inoculation an acute inflammation of the knee joint had been produced by the injection of a sterile irritant such as turpentine, alcohol, or quinine solution, the dissemination of the microorganisms was either inhibited or wholly prevented.

Rivers and Tillett (1925) confirmed these studies by finding that beef broth injected into the skin of rabbits protected against streptococci inoculated twenty-four hours later. No retardation was observed when the microorganisms were

thymol blue and is yellow or orange yellow to methyl red, its *p*H is probably in the neighbourhood of 6·0. For fuller discussion and instructions see: Britton (1942), & Clark (1928). A convenient comparator, complete with tubes of standard buffers with indicators, for the colorimetric determination of *p*H is obtainable from Messrs. British Drug Houses and Messrs. Hopkins & Williams. The B.D.H. Capillator set is a useful instrument for *p*H measurements of very small, as well as large, quantities of liquid.

SOLUTIONS

The indicators mentioned above may conveniently be purchased in ready-made solutions. If it is desired to prepare them from the solid dyes, then 0·1 g. of thymol blue, bromphenol blue, bromthymol blue or phenol red should be dissolved in 20 ml. of warm alcohol and diluted to 100 ml. with water. Preparation of Topfer's indicator is given on p. 111, and of methyl orange, methyl red and phenolphthalein on p. 141.

TABLE XII

PRESENCE OF B. PRODIGIOSUS IN RETROSTERNAL LYMPH NODES AFTER INTRAPERITONEAL INJECTION

Experiment	Interval between Injection of Irritant and That of Bacteria	Total Duration of Inflammation	Number of Colonies Recovered from Retrosternal Lymph Nodes	
			After Injection of Bacteria into Inflamed Peritoneal Cavity	After Injection of Bacteria into Normal Peritoneal Cavity
	hrs.:min.	*hrs.:min.*		
1	4:10	6 00	6	150
2	15·05	21:10	3	47
3	15:30	21:40	2	38
4	22·45	25:45	0	Innumerable
5	26:18	29.50	39	Innumerable
6	24.45	40:10	7	Innumerable

From Menkin, *J. Exp Med*, 1931, *53*, 647.

normal peritoneal cavity was much larger than the number of colonies from the nodes draining a peritoneal cavity inflamed by previous inoculation with an aleuronat suspension. The acute peritoneal inflammation evidently prevents the rush of injected bacilli to the tributary lymph nodes.

TABLE XIII

RETENTION OF B. PRODIGIOSUS AT SITE OF SUBCUTANEOUS INFLAMMATION INDUCED BY ALEURONAT

Experiment	Interval between Injection of Irritant and That of Bacteria	Total Duration of Inflammation	Number of Colonies Recovered	
			Inflamed Area	Normal Area
	hrs min.	*hrs.:min.*		
1	1 30	3·30	175	37
2	4.00	6.00	225	175
3*	4·10	6.00	250	125
4	26·00	30.00	115	8
5	26:18	29 50	50	6

* The site of inflammation was located in the peritoneal cavity, see Experiment 1, Table XII, for related data on retrosternal lymph nodes

Experiments were set up to determine whether this failure of bacterial dissemination to the tributary lymph nodes was actually due to retention of the microorganisms at the site of inflammation. As can be seen from the results recorded in

irritant is effective only when the inflammatory reaction has been going on in the cavity for 48 to 72 hours. Local fixation of substances at the site of inflammation, on the other hand, may be demonstrated within a relatively shorter time after the introduction of necrotizing inflammatory irritants (Menkin, 1929). The writer demonstrated, for instance, that trypan blue may be fixed as early as 30 minutes after the injection of an irritant (Table XIV, Experiment 4). With mild irritants, however, such as hemolytic streptococcus, fixation may be delayed for as long as two days (Menkin, 1933). The increased resistance to intrapleural inoculation of streptococci found by Gay and his collaborators is possibly the resultant of several factors. It is difficult, on the basis of available evidence, to refer the enhanced resistance to a single factor. Menkin has shown, for instance, that lymphatic blockade is a significant factor in evaluating resistance (1936c) but, in the process of local disposal of an infecting pathogen, there are doubtless other elements concerned. These may include the cellular constituents of an exudate, the type and quantity of cells, as well as the rapidity of their appearance at the site of inflammation.

Freund (1931) studied the capacity of young rabbits to fix bacteria at the site of inoculation. This investigator observed that, whereas pneumococci induce a severe inflammation at the site of inoculation in adult rabbits, young animals, on the other hand, fail to develop an appreciable local reaction. Concomitantly he found that death occurs in relatively few adult rabbits, but that young rabbits succumb with pronounced bacteremia.

Menkin likewise studied the behavior of various foreign inert substances injected into an inflamed area by direct observation of the tributary lymphatic nodes and vessels draining the area. Trypan blue, ferric chloride, colloidal iron, graphite particles, and a foreign protein were found to be readily retained at the site of an acute inflammation (1929, 1930, 1931b). Fixation of a dye could be demonstrated (as shown in Table XIV) within thirty minutes

The following are characteristic :—

Pigment	No of bands	Position on the spectrum. (Wave-length in $m\mu$)
Reduced haemoglobin	1	565
Oxyhaemoglobin	2	540, 578
Carboxyhaemoglobin	2	535, 572
Methaemoglobin	4	500, 540, 579, 630
Alkaline methaemoglobin	2	541, 580
Sulphaemoglobin	3	540, 578, 618
Haemochromagen	2	526, 557
Acid haematin	5	505, 540, 580, 638, 650 (in dilute HCl)
Alkaline hæmatin	1	607
Acid porphyrin	2	554, 600
Alkaline porphyrin	4	504, 539, 576, 622
Stercobilin (urobilin)	1	490

450 $m\mu$	500	550	600		700
Violet blue	blue green green	yellow yellow green	orange	r e d	

METHODS

Blood

Blood is diluted 1 in 5, or 1 in 10, with water, and cell membranes centrifuged out or allowed to settle. The clear solution is examined in a glass cell or tube. It is important that the greatest possible depth or concentration of solution (consistent with visibility) should be examined, and that a careful search should be made (with varying depths or concentrations of solution) for any bands in the red part of the spectrum (620–630 $m\mu$). The bands due to met- or sulph-haemoglobin, present sometimes in blood from patients treated with drugs (e.g., sulphanilamide and its derivatives), are not always easy to detect. If such bands (at approx. 620–630 $m\mu$) are seen, the solution should be treated with a drop of yellow ammonium sulphide. A band due to met-haemoglobin will then disappear; if sulphaemoglobin is present its band persists. For comparisons, laked blood may

TABLE XV

Presence of Horse Serum in the Blood Stream About 2 Hours after Intraperitoneal Injection

Experiment No.	Duration of Inflammation (Hours)	Animals with Inflamed Peritoneal Cavity — Dilutions 1 3	1 9	1 27	1 81	1 243	1 729	1 2187	Animals with Normal Peritoneal Cavity — Dilutions 1.3	1.9	1·27	1 81	1·243	1:729	1·2187	1 6561
1	24	Faint trace	Faint trace?	0	0	0	0	0	++	+	+	Trace	Faint trace	0	0	0
2	26	+++	++	+	+	Trace	Faint trace	0	+++	+++	++	+	Trace	Trace	+	+
3	26	++	++	+	0	0	0	0	+++	++	++	Trace	Faint trace	Faint trace	+	0
4	26	+	Trace	0	0	0	0	0	++	+	+	Trace	0	0	0	0
5	26	+	0	0	0	0	0	0	++	+	+	0	0	0	0	0
6	49	0	0	0	0	0	0	0	++	+	+	+	+	0	0	0
7	50	0	0	0	0	0	0	0	+++	++	+	Trace	0	0	0	0
10	49	+	Trace	0	0	0	0	0	++	+	+	Trace	0	0	0	0
11	50	++	+	Trace	0	0	0	0	++	+	+	Trace	0	0	0	0

From Menkin, J Exp Med, 1930, 52, 201

aleuronat). This prompt reaction allows a definite interval of time for the leukocytes to assemble for phagocytosis. The initial fixation of bacteria or of other injurious substances at the site of inflammation thus becomes a protective mechanism and plays a definite rôle in immunity.

ɪectroscope, may be quantitatively measured by means of τne Hartridge Reversion Spectroscope. In this instrument light from a source passes through a solution in such a way that two spectra are formed, one immediately above the other.

The spectra are also reversed with respect to one another, and the position of one of them can be altered longitudinally by moving a micrometer screw. If a glass cell filled with a solution of oxyhaemoglobin is placed in the path of the light the corresponding lines (α) in the spectra may be made to coincide (fig. 7). If, now, the cell is replaced by one containing some carboxyhaemoglobin, the bands will appear shifted with respect to one another (fig. 8). By adjusting the micrometer screw until coincidence is again obtained, the extent of the shift is measured and can be related to the percentage of carboxyhaemoglobin by reference to a graph, obtained as follows :—

Blood is diluted with ammonia solution (4 ml. of conc. ammonia per litre in water). The dilution of blood (usually 1 in 20) must be such that, with the cell used, the distance between the α and β haemoglobin bands is approximately equal to the width of one of them. The solution so formed is saturated with oxygen to give 100 per cent. oxyhaemoglobin and with coal gas to give 100 per cent. carboxyhaemoglobin solutions. The two cells A and B are then placed face to face so that the light traverses them both. Cell A is first filled with 100 per cent. oxyhaemoglobin and B with ammonia solution. A reading is taken at coincidence of the α haemoglobin bands.

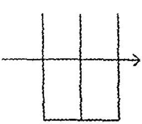


Fig. 9.

Cell A is now emptied and refilled with 100 per cent. carboxyhaemoglobin solution and another reading taken, at coincidence. For 50 per cent. saturation of carboxyhaemoglobin, cell A is filled with a mixture of equal volumes of dilute ammonia and 100 per cent. carboxyhaemoglobin solution and cell B with a similar mixture of ammonia and 100 per cent. oxyhaemoglobin solution. For

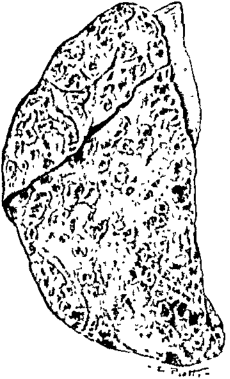


FIG. 32 The animal received 16 intravenous injections of ferric chloride solution The animal was killed and the lung placed in acidified potassium ferrocyanide. The Prussian blue reaction is limited to the caseous areas of tubercles. About actual size (From Menkin and Menkin, *J Exp Med.*, 1931, *53*, 919)

Chapter XII

TESTS OF FUNCTION

THE GLUCOSE TOLERANCE TEST

The patient is fasted for 12 hours or longer. The "fasting blood sugar" is estimated by the method described. Immediately after the blood has been taken, a solution of 50 g. of glucose in 250 ml. of water is given (for youthful subjects,

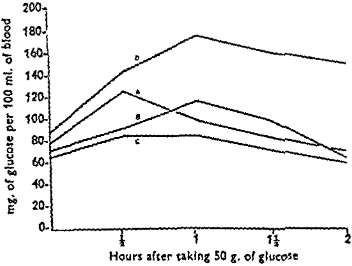


Fig. 11.—Glucose Tolerance Tests.

A & B. Normal.
C. Increased tolerance (e.g., Addison's disease).
D. Decreased tolerance (Diabetes).

the amount of glucose should be 1 g. per 3 lb. of body weight). Blood sugar estimations are then made at ½, 1, 1½, and 2 hourly intervals after the administration of the glucose. Samples of urine are taken before the test and 2–4 times during its course. In normal persons, the blood-sugar will not generally rise above 160 mg. per 100 ml., and the urine will not give a positive test for sugar. The blood glucose level

(1929) pointed out that bacteria, especially pneumococci, in the blood stream tended to localize readily in areas of cerebral softening. Burrows (1932) in a detailed monograph has adequately reviewed his observations as well as those of numerous investigators, on the localization in inflamed areas of bacteria, viruses, dyes, proteins, and inorganic particles. Fox (1936) has recently called attention to the more rapid localization of a dye in a younger lesion. This point has been essentially confirmed and extended in studies on xylol-treated areas by Rigdon (*Proc. Exp. Biol. and Med.*, 1939, *42*, 43).

Reference has already been made to the retention at the site of an acutely inflamed area of various inert substances (e.g. dyes, iron, graphite, foreign proteins) as well as of bacteria (Menkin, 1929, 1930, 1930*a*, 1931*b*). Intravenous injection of these materials is accompanied by their accumulation and concentration in inflamed areas owing to their inability to escape readily through draining lymphatics (Menkin, 1929, 1937*a*). The capacity of dyes or of other foreign substances to accumulate from the circulating blood in an area of inflammation has been utilized, as pointed out previously (cf. Chapter *III*), as a convenient method of detecting increased capillary permeability. The localization of trypan blue in inflamed areas has already been described in detail in an earlier section.

It seemed conceivable, in view of these various studies, that the development of the inflammatory reaction might be altered by the accumulation of various materials from the blood stream and their subsequent fixation in an area of injury.

The accumulation of iron injected into the circulating blood in inflamed areas. Since it had been found that iron was fixed *in situ* when injected directly into an inflamed area (cf. Chapter IX), studies were undertaken to determine whether, as in the case of trypan blue, the metal would accumulate in an inflamed area when introduced into the circulating blood stream.

25 mg. of ferric chloride in saline were injected intravenously. The areas of inflammation were caused by several

EXPERIMENTAL PROCEDURE

A solution of galactose (50 g. per 100 ml. of solution) is prepared and sterilized by filtration and steaming.

Graded Doses of Galactose. The patient receives no breakfast. An amount of solution equivalent to 0·5 g. of galactose per kg. of body-weight—e.g., 60 ml. of the 50 per cent. solution for a 60 kg. person—is injected intravenously from a 100 ml. syringe, the injection being given slowly over about five minutes.

Standard Dose of Galactose. The injection of 50 ml. of a 50 per cent. solution of galactose has given, with adults, results indistinguishable from those obtained with graded doses. This is a much more convenient amount to inject, because most hospital wards have 50 ml. syringes, whereas 100 ml. syringes are usually special equipment.

Samples of Blood. The first sample of blood is taken immediately after the administration of the galactose (5-minute sample), the second sample at ½ hour, and further samples at 1, 1½, and 2 hours. A sample taken before the galactose administration may be substituted for that at 5 minutes. This resting sample is a useful check on the analytic method because it should contain no galactose.

Analysis. The samples of blood (0·2 ml. of capillary blood) are washed into centrifuge tubes containing 2·2 ml. of isotonic sodium sulphate and 0·8 ml. of 10 per cent. sodium tungstate. The estimation is completed as described on p. 23.

RESULTS

The galactose values are plotted against time and for a normal person usually give a curve which begins about 200 mg. of galactose per 100 ml. of blood, falls steeply during the first hour, and reaches a figure between 0 and 10 mg. per 100 ml. at the end of two hours. In most cases of obstructive jaundice the curve of blood galactose follows the same course, there being little or no galactose left in the blood at the end of two hours. In conditions of liver damage the level of galactose in the blood does not

TABLE XVI

AMOUNT* OF IRON IN INFLAMED AREAS ON ANIMALS THAT RECEIVED NO IRON AND OF ANIMALS INJECTED WITH FERRIC CHLORIDE

EXPERIMENT	INJECTED ANIMALS		NON INJECTED ANIMALS	
	Normal Skin Areas	Inflamed Skin Areas	Normal Skin Areas	Inflamed Skin Areas
1	9.3	13 0	8.4	9.2
2	12.5	15.7	8.4	9 4
3	7.7	15 5	5 6	9 6
4	16.6	29.6	10 9	9.0
5	6.9	10.6	7.9	11.2
6	9.7	12.7	9.2	9.9
Average	10.4	16 2	8 4	9 7

* Figures are expressed in milligrams of iron per 100 grams of dry tissue
From Menkin, *J. Exp. Med*, 1930, *51*, 879

inflamed areas of an animal not injected with this metal is contained in a form which does not give the Prussian blue reaction. This accounts for the negative reaction when the qualitative test is applied in these animals to inflamed areas of relatively short duration.

These experiments suggest that fixation of a metal or dye in an inflamed area may have clinical application. It is possible that one might find practical uses for a substance which, by accumulating from the blood stream would enhance the roentgen appearance of various inflammatory lesions. *It is conceivable that dyes, iron-containing substances,* and various other materials by their concentration at the site of inflammation may alter the character or course of the inflammatory reaction.

THE ACCUMULATION OF IRON IN TUBERCULOUS AREAS

In connection with the foregoing observations on the accumulation of iron in inflamed areas, experiments were set up to determine whether iron in the form of ferric chloride is capable of penetrating the tubercles of rabbits. It was thus demonstrated that repeated intravenous injections of this *iron salt* are followed by the deposition of the metal in tubercles of the lungs (Menkin, 1930*b*; Menkin and Menkin, 1931).

however, and should only be interpreted against the background of the clinical and other biochemical findings.

INTRAVENOUS HIPPURIC ACID TEST

PRINCIPLE

The detoxicating power of the liver is assessed by administering benzoic acid (as the sodium salt) which is conjugated with glycine, in the liver, to form hippuric acid, and excreted as such by the kidneys. The specificity of the test for hepatic as opposed to renal function is not yet certain, and a simultaneous urea clearance test should be run in parallel.

METHOD

Administration.—Twenty ml. of an 8·85 per cent. solution of sodium benzoate, = 1·77 g. sodium benzoate, equivalent to 1·5 g. benzoic acid, is injected slowly into an arm vein. Immediately after the injection, and exactly one hour later, the bladder is emptied. The first specimen is discarded, and the hourly specimen used for the analysis. In order to ensure an adequate urine output, the patient is given a pint of water to drink after the injection.

Estimation (Weichselbaum and Probstein, 1939).—The urine volume is measured, and if it exceeds 120 ml. is acidified with a little glacial acetic acid, evaporated down and again measured. If the urine is very heavily pigmented, a little charcoal is added. The warm urine is then saturated with salt, 30 g. per 100 ml., heated and filtered. The filtrate is acidified with 50 per cent. sulphuric acid, till acid to Congo Red; and is left to stand in the cold, preferably overnight, until crystallization is complete. Crystallization can sometimes be induced by scratching the sides of the vessel with a glass rod, or by adding a small crystal of hippuric acid. The crystals are then filtered off by suction, washed with cold 30 per cent. sodium chloride and dissolved in distilled water by warming. The solution is titrated against 0·5 N-sodium hydroxide, using phenolphthalein as indicator.

TABLE XVII

SUMMARY OF STUDIES ON EFFECT OF FERRIC CHLORIDE IN TUBERCULOUS RABBITS

Series	Results Published in	Dose of Virulent Tubercle Bacilli	Route of Inoculation of Virulent Bacilli	Total No of Rabbits	Aver. Survival Time		Increased Survival Time Exp. Animals
					Control Animals	Exp Animals	
		mg.			*days*	*days*	%
1	1932	.001	Intrav.	16	61	109	78
2	1933	.001 (*appr.*)	"	20	94	135	44
3*	1936	.01	"	19	84†	133	58
4	1934	.05	Subcut.	10	130	246	89
5‡	1934	.05	"	10	81	198	144
Aver.					90	164	82

Series 6 (1933) 16 tuberculous rabbits sacrificed between 45th and 79th day of the disease. Extent of tuberculous involvement in lungs of experimental animals considerably less than in controls [illegible] the disease. [illegible] retardation [illegible]

longation of survival time in five independent series of experiments, *each of which was essentially confirmatory of the* preceding one, was 82 per cent.

The combined form of treatment, consisting of preliminary vaccination followed by ferric chloride treatment, enhanced the effect of the iron salt. This is illustrated *in series 5 of* Table XVII showing an increased survival time of 144 per cent. This was attained by preliminary vaccination with a Cernay strain of bacilli obtained from the Pasteur Institute. Vaccination with the bacillus Calmette-Guerin (B.C.G.) likewise enhanced the chemotherapeutic effect of ferric chloride. In this particular series the reinfecting dose of bacilli (Ravenel bovine strain) was 10 times greater (.01 mg.) than that employed in previous sets of intravenously infected rabbits. The purpose of this was to determine whether the favorable effect of ferric chloride on the experimental disease would still be appreciable with massive infection. To avoid hemolysis, the iron chloride solution was rendered isotonic

urine is above 2 ml. per minute) is usually about 40 per cent. greater than the "standard" clearance (flow of urine less than 2 ml. per minute). The average normal values are as follows :—

> For the "maximum clearance" an average of 75 ml. of blood per minute with variations from 64 to 99 ml., and for the "standard clearance" an average of 54 ml. with variation from 40 to 68 ml.

Nephritic patients with diminishing renal efficiency show a decreased "clearance" (down to about 40 per cent.) before the blood urea and the blood creatinine begin to rise; on the other hand, the maximum specific gravity of the urine is often diminished before the urea clearance test gives abnormal results. When the clearance falls to 5 per cent. the symptoms of uraemia usually appear.

METHOD

The patient is given no breakfast and nothing to drink in the early morning other than water if desired.

The test is carried out in the forenoon as follows :—

0 hours :	Empty bladder Give 15 grams urea in 500 ml. water, if the blood urea is not raised. (If blood urea is known to be raised, or likely to be raised, give 500 ml. water only.) *		No.
½ hour :	Take blood for urea estimation . .	Blood	1
1 hour :	Empty bladder † — send complete specimen to laboratory . . .	Urine	1
1½ hours :	Take blood for urea estimation . .	Blood	2
2 hours :	Empty bladder † — send complete specimen to laboratory . . .	Urine	2

* The patient's blood urea should usually be determined a day or two before

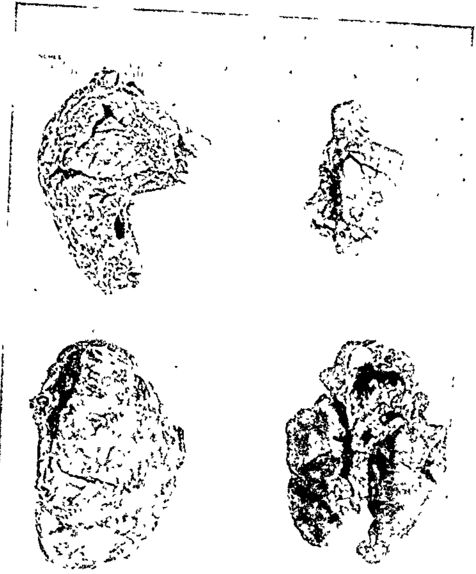


FIG. 33. Cf. series 5, Table XXIII. Comparison of lungs of Rabbit 6-28 and Rabbit 6-21 on left [illegible]

vaccina[illegible]

mental [illegible]

The amount of tuberculous involvement is scant in comparison with the extensive confluence of lesions in the controls. (From Menkin, *J. Exp. Med*, 1934, *60*, 463)

usually by injection of a small amount of histamine. This may be called "the pharmacological stimulus."

Histamine Test

Histamine has definite advantages over the ordinary test meal. It evokes a maximum response, and often produces acid secretion where the ordinary test meals fail completely. It is independent of the psychic factors involved in the taking of any ordinary meal. It adds nothing to the stomach so that a pure juice, undiluted and uncontaminated, is obtained; and no neutralization of the acid can take place through food constituents.

The Combined Alcohol and Histamine Tests

Alcohol as a physiological stimulant has several advantages over gruel, charcoal biscuit or dry toast. It is much less objectionable to take, is easily swallowed, and evokes a feeling of pleasure instead of distaste on the part of the subject.

The gastric juice obtained is ideal for analysis. It is thin and easily pipetted. No suspended food material obscures the colour and renders it turbid. The presence of bile, mucus, and altered blood are much easier to detect, and the end-points in the titration are sharper and better defined.

Alcohol seems to cause a more ready response of gastric secretion than the other test meals. The curve of acidity rises more sharply. There is not the preliminary drop following the resting specimen which is observed in most fractional test meals, and is probably due to the neutralization of the first juice secreted, by the food stuff administered. Alcohol does not neutralize any of the acid. It is a neutral substance and only dilutes the juice. Further, a sufficient amount can be given in much less volume so that the dilution factor is small. Instead of a pint of gruel, only 50 ml. of aqueous alcohol are usually given.

The response to alcohol is much more quickly over—partly because it does not remain so long in the stomach. The

TABLE XVIII

IRON CONTENT * IN ORGANS OF TUBERCULOUS RABBITS (NON-INJECTED CONTROL ANIMALS)

Rabbit No.	Duration of Disease (Days)	Spleen: Extent of Tuberculous* Involvement	Spleen: Iron† Content	Liver: Extent of Tuberculous* Involvement	Liver: Iron† Content	Bone Marrow (Femoral): Extent of Tuberculous* Involvement	Bone Marrow (Femoral): Iron† Content	Lungs: Extent of Tuberculous* Involvement	Lungs: Iron† Content	Kidney: Extent of Tuberculous* Involvement	Kidney: Iron† Content
12-11	108	0			109.0	Trace	90.6	++++	33.6	+	25 6
12-38	127	0	92.2	0	50.2	+	61.9	++++	21.9	+++	45.1
12-54	97	0	700 0	0	84 1	0		++	52.0	+	31.7
12-49	90	+		0				+++	33.1	++	46.9
12-51	151	0	403 5		97 5	0	120 2	++++	25 3	++	34.4
12-48	88	0	318.8	0	57 9	0		+++	20.9	+++	49 3
12-50	100	++		0	56.6			+++	25.8	+++	28.7
2-04	79							++	26.4		
14-37	91‡	Trace		0		0		+++	15.8	+++	
14-19	70	0		0		0		++	36.1	++	
14-99	84‡	++		0		0		+++	24.4	+	
14-97	71‡	0		0		0		+	21.7	Trace	
14-31	77‡	0	168 9	0	42.6	0		++	23.8	+	27.6
4-20	124	0		0				++	49.5	++	

acid usually turns out to be organic acid; a strong test for lactic acid is found. Blood is frequently present, and mucus is excessive. The foul smell of the resting juice is characteristic.

2. *Gastric Ulcer.* The results are very irregular, but are usually fairly like the normal. The curves for free and total HCl are in many cases within the normal range, although high results are often encountered. Mucus and bile may or may not be present. The presence of altered blood—"coffee-grounds"—is significant.

Stenosis is evidenced by an increasing acidity which does not show the normal tendency to fall, giving a "plateau" type of curve. The presence of blood with an increasing acidity curve indicates a gastric rather than a duodenal ulcer.

No differentiation can be made on the basis of the curve between a simple hyperchlorhydria and one due to an organic lesion. Only the presence of blood will indicate the latter.

3. *Duodenal Ulcer.* Hyperchlorhydria is usually present; it may be very marked. If the ulcer be just below the pylorus, stenosis may result. The acid curve will then be very high and will not fall for some time. Other findings are not of note.

4. *Achlorhydria.* True achlorhydria is found in pernicious anaemia. No secretion is brought out by either the alcohol or the histamine.

Apparent achlorhydria, on the other hand, shows an absence of free HCl in the alcohol part of the test, but the histamine successfully stimulates its secretion.

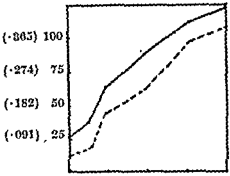
5. *Gastro-enterostomy,* if successful, relieves hyperchlorhydria completely. The free HCl is very low or absent, due to the constant regurgitation of bile. The curve of total acidity is a low normal.

6. *Partial Gastrectomy.* The curve of free acidity is low. Bile is frequently present. The stomach empties quickly. If the operation has been unsuccessful the acidity curves will continue to be high. A routine test meal should be conducted within a few weeks of the operation.

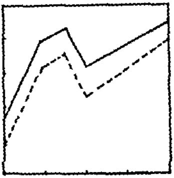
TABLE XIX

IRON CONTENT * IN ORGANS OF TUBERCULOUS RABBITS INJECTED WITH FERRIC CHLORIDE

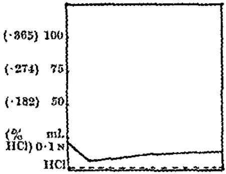
Rabbit No	Duration of Disease at the Time of Observation (Days)	Amount of 0 25 Per Cent Ferric Chloride Injected (cc)	Spleen		Liver		Bone Marrow (Femoral)		Lungs		Kidney	
			Extent of Tuberculous* Involvement	Iron† Content	Extent of Tuberculous* Involvement	Iron† Content	Extent of Tuberculous* Involvement	Iron† Content	Extent of Tuberculous* Involvement	Iron† Content	Extent of Tuberculous* Involvement	Iron† Content
12-59	110	135	0	448 2	0	246 3	0	507.8	+	66.2	++	36 1
12-19	92	135	0		0	139.2	+		++	61.1	Trace	33.1
12-29	88	123					++		+++	63.1	+	104.6
12-52	125	135		1032.2		297.6	+	348 2	+ to ++	165.1	+	97.6
12-39	164	153	0	310 0	0	184 0	0	77.1	+	52 3	+	25 3
12-42	92	141	0		0		0		++	35.7	+	
2-07	65	142							++	91 26		
2-12	41	57							++	58 87		
14-40	72‡	132	0	231.7	0	90.83	0		+++	16.25	++	34.8
14-17	76‡	144	Trace	338 8	0	124 9	0		+++	15.5	+++	31.6
14-21	83‡	162	0	653.87	0	144.4	0		++	36 5	+	26.8
14-18	92‡	186	0		0		0		Trace to +	47.4	Trace	
14-26	69‡	120		689 5			0		Trace	48.7	+	39.4
3-09	184	526	0		0		0		+	152.0	Trace	
3-25	90	280	0		0				+	103.1	Trace	



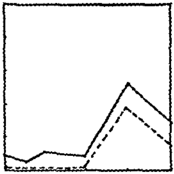
Duodenal ulcer; resting juice normal: marked response to stimuli in free and total HCl; slow emptying (plateau type); bile present, no blood.



Duodenal ulcer; free and total HCl rather high; no mucus, bile or blood.



Pernicious anaemia: no free HCl; low total; no starch; mucus and small amount of bile present.



Apparent achlorhydria (gastric ulcer); little mucus and bile; no blood.

Fig. 13.

or lung tissue of experimental animals when compared with those of control rabbits.

M. A. Kadish and the writer studied the quantitative distribution of iron in various tissues of ferric-chloride-injected tuberculous rabbits and of non-treated tuberculous rabbits. The amount of iron recovered in liver, spleen, bone marrow, lung, and kidney was compared with the extent of tuberculosis found at necropsy in these various organs. The data are summarized in Tables XVIII and XIX.

The results fail to show an absolute parallelism between the amount of iron and the degree of tuberculosis. There are, however, several suggestive correlative features. The liver, spleen, and bone marrow reveal at autopsy practically no tuberculous involvement. This, especially in view of the studies of Lurie (1928, 1929), is not unexpected in tuberculous rabbits. These three organs contain large amounts of iron. On the other hand, both lungs and kidneys reveal extensive tuberculosis. Their iron content is, curiously enough, relatively low (Table XVIII). The repeated intravenous injections of ferric chloride induce a marked rise of over 100 per cent in the iron of the liver, bone marrow, and lungs. The extent of pulmonary involvement in the experimental animals seems somewhat less prominent than in the control group (cf. Table XVIII with Table XIX). And yet, although the iron level in the lungs of the experimental rabbits became doubled, i.e. reaching an average level of about 66 mg. per 100 grams of dried tissue, tuberculous lesions remained conspicuous. In the liver of control animals, on the other hand, the average iron content was found to be 74 mg. per 100 grams of dried tissue. The iron content of the lungs in the experimental group is therefore approximately of the same magnitude as that of the liver in the controls. Nevertheless, the high incidence of tuberculous lesions in the former is in striking contrast with their absence in the latter. In brief, these facts definitely indicate that derangement of iron metabolism is most likely not the sole factor conditioning the proliferation or destruction of tubercle bacilli in tissue. It is

less than 20 ml. Suppose, for example, it is 18·45 ml.; then every 18·45 ml. of the acid is equivalent to 20 ml. of N-acid. That is, 18·45 ml. of acid must be diluted with water to 20 ml., to give N-acid. An appropriate dilution of part or all of the remaining approximately N-acid is now made.

The mixed solution should be exactly N, and may be again tested against sodium carbonate to confirm this.

NORMAL SODIUM HYDROXIDE

Sodium hydroxide ($NaOH = 40$) *contains in 40 g. of substance* an amount of sodium (23 g.) which is equivalent to 1 g. of hydrogen. This "equivalence" of sodium to hydrogen is best understood by reference to the equation—

$$HCl + NaOH = NaCl + H_2O.$$

Approximately 41 g. of the solid are weighed out as quickly as possible, and put into 50 ml. of water in a litre flask. When cold, the solution is mixed and made to volume. This approximately N-solution is standardized against pure oxalic acid.

$$2NaOH + \underset{90}{\begin{matrix} COOH \\ | \\ COOH \end{matrix}} \longrightarrow \begin{matrix} COONa \\ | \\ COONa \end{matrix} + 2H_2O$$

Hence, 2 litres of N-sodium hydroxide are equivalent to 90 g. of anhydrous oxalic acid, or $90 + 36 = 126$ g. of oxalic acid crystals, $\begin{matrix} COOH \\ | \\ COOH \end{matrix} .2H_2O$

Thus,

1·26 g. of oxalic acid crystals

$\equiv$ 20 ml. of N-sodium hydroxide.

Therefore about 1·2 g. of oxalic acid are accurately weighed out, washed into a flask, and the solution titrated with the approximately N-sodium hydroxide, with methyl red

solution in concentration of 0.0625 per cent. No untoward *systemic* deleterious effects attributable to the iron have ever been observed in any of these patients. In the opinion of the writer, rigidly controlled and cautiously carried out investigations on early or only moderately advanced cases of human pulmonary tuberculosis should be undertaken in order to determine whether or not this iron salt will yield favorable effects similar to those found on tuberculous rabbits. It is believed that the present facts warrant such an investigation.

The Accumulation of Bacteria at the Site of Inflammation

Experiments were undertaken to determine whether bacteria, that are fixed *in situ* when injected directly into an inflamed area, would accumulate rapidly at the site of inflammation when injected into the blood stream (Menkin, 1931*b*).

Two or three areas of inflammation were induced by the injection of 0.5 cc. concentrated broth into the skin of the abdomen of rabbits. After a short interval of time 3 to 7 cc. of saline suspension of either *B. prodigiosus* or, in two experiments (Nos. 5 and 6, Table XX), *B. pyocyaneus* was injected intravenously. After several hours the animal was killed with ether. The skin of the abdomen was treated first with 95 per cent, then with 70 per cent alcohol, and finally with sterile distilled water. Inflamed and normal skin areas were removed and weighed separately in sterile petri dishes. An extract of each area was made by grinding it in a mortar with a volume of saline equal to twice its weight in grams. Agar tubes were inoculated with these extracts. The viable bacilli were evaluated by counting the individual colonies.

The numbers of colonies recovered from inflamed and normal areas are shown in Table XX. It is clear that the number of intravenously injected bacteria accumulating at the site of inflammation is distinctly greater than in normal skin areas. These observations are in agreement with results

TENTH NORMAL SODIUM THIOSULPHATE

Sodium thiosulphate ($Na_2S_2O_3$) reacts with iodine as follows:

$$2Na_2S_2O_3 + I_2 \longrightarrow 2NaI + Na_2S_4O_6$$

Hence, 1 gram mole of thiosulphate ≡ 1 gram atom of iodine and (since iodine reacts with hydrogen to give hydriodic acid, HI) 1 gram mole of thiosulphate is contained in 1 litre of N-solution. Approximately 0·1 N-sodium thiosulphate is made by dissolving 25 g. of the crystals ($Na_2S_2O_3 . 5H_2O$ = 248·2) in water, and making to 1 litre.* This solution is then standardized against a known amount of iodine, obtained from potassium biiodate ($KIO_3 . HIO_3$) solution by the reaction—

$$\underset{889 \cdot 97}{KIO_3 . HIO_3} + 10KI + 11HCl \longrightarrow 11KCl + 6I_2 + 6H_2O$$

0·1 N-potassium biiodate is therefore made by dissolving $\frac{889 \cdot 97}{10 \times 12} = 8 \cdot 250$ g. of pure potassium biiodate in water in a litre flask, and making to volume. The titration is carried out as follows:—

A flask containing a solution of approximately 1 g. of potassium iodide in a little water is treated with about 10 ml. of approximately 5 N-hydrochloric acid (made by 50 per cent. dilution with water of the concentrated acid). Twenty-five ml. of the 0·1 N-potassium biiodate solution are then run in from a pipette. The iodine set free is titrated with the approximately 0·1 N-sodium thiosulphate, until a light yellow colour is obtained. Two drops of starch indicator are added, and the titration continued until the solution is colourless. The titration should be less than 25 ml. Dilution of the thiosulphate to exactly 0·1 N is carried out as in the previous cases.

Starch Indicator.—One hundred ml. of water are heated in a beaker to boiling. A paste of approximately 1 g. of starch in a little cold water is poured into the beaker. A few crystals of phenol red are added, and boiling is continued for a few minutes. The solution is cooled and preserved with a little chloroform.

* 1 ml. of 20 per cent. sodium carbonate and 10 ml. of amyl alcohol per l. may be included in the solution as a preservative.

TABLE XXI

THE ACCUMULATION OF HORSE SERUM IN INFLAMED CUTANEOUS AREAS*

Experiment No.	Inflamed Skin: Dilutions 1:3	1:9	1:27	1:81	1:243	1:729	1:2187	Normal Skin: Dilutions 1:3	1:9	1:27	1:81	1:243	1:729	1:2187
21	+	+	Trace	Faint trace	0	0	0	Faint trace	0	0	0	0	0	0
22	++	+	Trace	0	0	0	0	Trace	0	0	0	0	0	0
23	+	Trace	0	0	0	0	0	Trace	0	0	0	0	0	0
24	+++	++	+	Trace	0	0	0	+	Trace	Faint trace	0	0	0	0
25	++	+	+	Trace	0	0	0	+	+	Trace	0	0	0	0
26	++	++	+	Trace	0	0	0	++	+	Trace	0	0	0	0
27	++	++	+	Trace	0	0	0	+	+	Trace	0	0	0	0
28	+++	++	+	Trace	0	0	0	++	+	0	0	0	0	0
29	+++	++	+	0	0	0	0	++	+	+	0	0	0	0
30	++	+	+	0	0	0	0	+	Trace	0	0	0	0	0

* The accumulation of the foreign protein in the skin was followed by extracting the tissue area with saline and then utilizing the precipitin test with appropriate antiserum.

From Menkin, *J Exp Med*, 1930, *52*, 201

TENTH NORMAL IODINE SOLUTION

Approximately 13·5 g. of pure sublimed iodine are dissolved in a solution of 24 g. potassium iodide in about 200 ml. of water in a litre volumetric flask. The solution is diluted to the mark, mixed, and standardized against 0·1 N-sodium thiosulphate.

INDICATOR SOLUTIONS

Methyl Orange.—0·1 g. of methyl orange is dis 100 ml. of water.

Methyl Red.—0·2 g. of methyl red in 100 ml. of al

Phenolphthalein.—0·5 g. of phenolphthalein is diss 50 ml. of alcohol and 50 ml. of water are added.

blood stream in the inflamed tuberculous lesion and, by its presence there, induce an enhanced local inflammatory reaction (Menkin, 1930*a*).

Sanarelli (1924) described the reaction which occurs when *a preliminary intravenous injection of cholera vibrio* is followed 24 hours later by an intravenous injection of a filtrate of colon bacilli. The animals (guinea pig or rabbit) succumb revealing at post-mortem examination an acute hemorrhagic inflammation of the gastro-intestinal tract, extreme congestion of the visceral organs, and an acute inflammation of the kidneys. Shwartzman (1928) described a similar cutaneous phenomenon with bacterial filtrates. This investigator injected the filtrate of *B. typhosus* into the skin of a rabbit. Twenty-four hours later when the same filtrate or a filtrate of a non-related organism, such as the meningococcus, is introduced into the circulating blood, hemorrhagic necrosis frequently appears at the site of the cutaneous injection. Hanger (1928) obtained essentially similar results with filtrates of *B. lepisepticum.* Menkin (1931), on the basis *of his studies on the localization from the blood stream of foreign proteins at the site of an acute inflammation, suggested that the intense cutaneous reaction following the intravenous injection of a bacterial filtrate may be in part the result of a concentration of this substance in an area of the skin already inflamed.* The reaction does not have to take place with all bacterial filtrates or with all inflammatory irritants. The extent of induced local vascular thrombosis, the degree of permeability of the capillaries, and the optimum of the synergistic action of two irritating substances on one another when administered by two different routes may modify the morphological appearance of the final reaction. *Such an interpretation of the phenomenon as an enhanced non-specific inflammatory reaction* has been in large measure *supported by the studies of* Debonera, Tzortzakis, and Falchetti (1932), Freund and Smith (1934), Sickles (1931), Karsner and Moritz (1934), Freund (1934), and more recently by Moritz (1937). Bordet (1936) *classifies the* Sanarelli-

(millimeters of solution) will be minimum; and the depths of two different solutions of the same coloured substance should be in inverse ratio to the strengths of the solutions. Generally speaking, absorption will be found to be maximum for red solutions in the green or blue-green, and conversely, green solutions will show maximum absorption in the red. Blue and violet solutions are maximally absorbing in the yellow, orange, and red; and yellow and orange solutions in the blue and violet.

The grey screen of an appropriate density, together with the light filter showing maximum absorption, may be used as a permanent standard for any colorimetric method. It should be calibrated against the coloured solution of known strength — the "standard"; and the general equation for calculating the result for an unknown solution—the "test" —then becomes:—

$$\frac{\text{Read. of standard against grey screen}}{\text{Read. of test against grey screen}} \times \text{Conc. of standard} = \text{Conc. of test.}$$

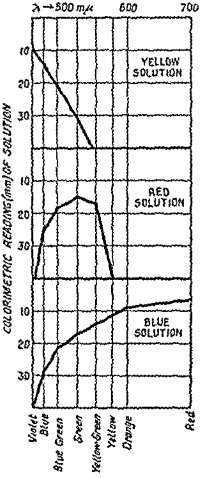


FIG. 14.

Absorption curves of standard solutions (neutral grey screens, spectral filters). Yellow solution: nesslerized ammonium chloride solution (strong urea standard); neutral screen, density 0·75. Red solution: bilirubin standard, 0·1 mg. in 25 ml. (*Haslewood and King*, 1937); neutral screen, density 0·50. Blue solution: uric acid standard; neutral screen, density 0·50.

The colour of any solution may also be expressed as its "extinction coefficient," (E). The calculation is as follows:

$$E = \frac{\text{Density of grey screen (e.g., 0·50)}}{\text{Reading of solution against grey screen (in cm.)}}$$

examination (Menkin, 1933, 1935). An increase in the functional filtration through the capillary endothelium, as indicated not necessarily by morphological evidence, but rather by the ability of foreign materials or bacteria from the blood stream to accumulate in the prepared area, would perhaps explain the Sanarelli-Shwartzman phenomenon as a peculiar manifestation of a non-specific inflammatory reaction induced by the superimposition of an irritant that concentrates from the circulating blood in a previously inflamed area. In this connection it is, however, to be noted that Bordet (1936) was unable to detect any accumulation of trypan blue in the prepared skin area when he substituted an intravenous injection of the dye for the filtrate on the day following the cutaneous inoculation. On the other hand, Moritz (1937) reported that when a local state of hypersusceptibility was elicited by the subcutaneous injection of *B. aertrycke* filtrate, the subsequent intravenous injection of living *B. aertrycke* led at the prepared site to the development of a hemorrhagic necrosing inflammation in which the bacteria localized.

Summary. Injury to tissue may determine the localization of foreign substances, bacteria, or ultrafilterable organisms present in the circulating blood.

Ferric chloride in dilute concentration, injected into the circulating blood of rabbits, rapidly accumulates in areas of acute inflammation. It is to be recalled that the direct introduction of this salt into an acutely inflamed area is followed by retention of the metal *in situ*, thus preventing it from reaching the tributary lymphatic vessels and nodes as readily as under normal circumstances.

Repeated intravenous injections of ferric chloride in tuberculous rabbits are followed by an accumulation of iron in tubercles. The localization is particularly conspicuous within the foci in the lungs. There is a corresponding rise in the iron level of the lung tissue of over 100 per cent. Repeated injections of ferric chloride also induce a marked augmentation of the ferruginous content of the liver, spleen, and bone

measurement, and the elimination of eye fatigue and personal error.

CONSTRUCTION

The construction and assembly of the instrument are illustrated in fig. 15. An ordinary surgical head-lamp furnishes the source of light (E). The bulb is activated from an accumulator or constant voltage transformer. A steady, non-fluctuating light is obtained. Immediately in front of the lamp is mounted a block of wood (well-seasoned teak or oak 3 × 3 × 2 in.) which has been bored vertically to fit a $\frac{5}{8}$-in. test-tube (A) and a $\frac{1}{2}$ × 2-in. metal bolt (B) whose nut is countersunk in the block. The bolt, which should fit its hole tightly, is used to vary the size of the slot through which the light passes. The slot is made by boring a $\frac{1}{2}$-in. hole from front to back, (passing through the vertical holes) and then sawing up from the bottom of the block to make the slot $\frac{1}{2}$ in. wide by $1\frac{1}{2}$ in. high. At the front of the block a groove is made 1 in. wide and $\frac{1}{8}$ in. deep to carry a light filter (D). A similar groove $\frac{7}{8}$ in. wide is made at the back of the block to fit the photoelectric cell (C). The block is mounted on a base board of wood, and the $\frac{5}{8}$-in. hole is extended $\frac{1}{4}$ in. into this so that the end of the test-tube drops below the bottom of the slot.

The photoelectric cell is of the selenium type, 22 × 40 mm. The " EEL " electroselenium cell supplied by Messrs. Evans of Bishop's Stortford has proved satisfactory. A copper wire is fitted on the surface of the rear groove in suitable position to make contact with the exposed strip of selenium which lies near the edges of the front surface of the cell. The wire is connected to an ordinary electric terminal screwed into the back of the block. A lead from this front-surface terminal connects with the negative pole of the galvanometer. A second terminal carries a spring clip which holds the photoelectric cell in place and makes contact with its back surface. The back-surface terminal is connected to the positive pole of the galvanometer.

Light filters of glass have been used. The Chance red (OR 2), green (OGr 1), and blue-green (OB 2) glass filters have been found suitable for most purposes. Alternatively, Ilford

CHAPTER XI

ALLERGIC AND ANAPHYLACTIC INFLAMMATION

Specific inflammation in the immune animal is the altered type of reaction induced by reinfection with either homologous bacteria or protein. For the sake of convenience, three types of response will be considered: 1) the allergic inflammation as exemplified in tuberculosis, 2) the fixation of pyogenic bacteria in immune animals, and 3) the anaphylactic inflammation as characterized particularly by the so-called Arthus phenomenon (local anaphylaxis).

Allergy and Immunity in Tuberculosis

Koch (1891) discovered an important principle in regard to the mechanism of immunity in tuberculosis when he described the reaction that takes place when a tuberculous guinea pig is reinoculated with either living or dead tubercle bacilli. This reaction to reinfection is termed the Koch phenomenon. In brief, Koch found that within ten to fourteen days after a healthy guinea pig is inoculated with a pure culture of tubercle bacilli, a hard nodule appears at the site of inoculation; this nodule ultimately breaks down and ulcerates. At the same time the tributary lymph node becomes enlarged and caseous. An entirely different picture develops when a tuberculous guinea pig is reinoculated four to six weeks following the primary infection. An acute inflammatory reaction occurs within a few hours at the point of reinjection without involving to any significant extent the satellite lymph nodes. Pirquet (1906) proposed the term "allergy" for the altered response of tuberculous tissue to reinfection. The tuberculin reaction, which may be regarded as a manifestation of the Koch phenomenon induced by tuberculo-protein, is histologically an acute inflammation.

100 will correspond to percentages of light transmission which are less than 100 per cent. of the incident light. They can be thought of as different degrees of extinction of the incident light. The extinction E or "optical density" is defined as the logarithm of the ratio of incident to transmitted light—

i.e., $E = \log. (100/\% \text{ transmitted light})$.

If two solutions of different optical density are interposed in the light path, then their extinctions E will be in the same relation one to the other as their concentrations of pigment —i.e., $E_1/E_2 = C_1/C_2$. This simple photometric expression of Beer's law defines the condition to be aimed at with a *photoelectric colorimeter. To simplify the reading of E,* and hence to simplify the calculation, the logarithmic scale is used on the galvanometer. It is arrived at in the following way. If, for instance, a coloured solution transmits only 50 per cent. of the incident light—i.e., gives a reading of 50 on the linear scale—then its extinction $E = \log. 100/50 = 0{\cdot}301$. The point opposite 50 on the linear scale is marked 0·301 on the logarithmic scale. Similarly, 100 on the linear scale is 0 on the logarithmic, 10 on the linear is 1 ($\log. 100/10 = 1$) on the logarithmic, and so on. For convenience, the values of E, which comprise the logarithmic scale, are multiplied by 100, and are plotted as whole numbers. The extinction E of a coloured solution is spoken of as the "extinction coefficient" when it is determined at, or reduced to, a solution depth of unity—i.e., 1 cm.

OPERATION OF THE INSTRUMENT

The galvanometer is levelled, the suspension is released and the "spot" or pointer adjusted to the ∞ mark at the left of the logarithmic scale (0 on the linear scale) by turning the suspension knob. The light is now switched on. The bolt is turned up or down to regulate the amount of light reaching the photoelectric cell, until the needle is at 0. It should remain at this point and will do so after the first few minutes if the electric supply is taken from a properly charged accumulator or adequate transformer.

might be interpreted on the basis of the well-established fact that there occurs a much greater destruction of tubercle bacilli in immune animals than in normal ones." As pointed out by the writer (1931), an interpretation of this sort is difficult to accept in view of the facts obtained. Willis (1925) demonstrated that the regional lymph nodes in immune animals were infected at the end of two weeks after cutaneous inoculation. If the apparent retardation of bacilli at the site of inoculation was primarily due to their destruction *in situ*, their appearance after a latent period of two weeks in the regional lymph nodes would be difficult to explain. Furthermore, Menkin (1931*b*) showed that an acute inflammatory reaction caused by a non-specific irritant prevented for at least several hours the dissemination of *B. prodigiosus* to the tributary lymph nodes. A distinctly larger number of microorganisms was recovered from the site of inflammation than from the corresponding normal control area. These experiments proved that an acute inflammation *per se* retards the dissemination of bacteria into the regional lymphatics. Rich and McKee (1934), in a discussion of some of these results, readily agreed that an acute inflammation is of assistance in preventing the dissemination of bacteria. However, they correctly pointed out that this phenomenon is only exhibited when the inflammatory reaction has attained sufficient intensity to induce a walling-off of the affected area. In other words, on the basis of Rich's view, the observations of Issayeff (1894) and of many other investigators, including the writer, fail to explain the immediate immobilization which takes place upon reinoculation of an immune animal. The studies of Menkin (1933) on bacterial invasiveness (see Chapter XIII), however, demonstrated that the rapidity with which an area of acute inflammation becomes walled off so that foreign substances fail to spread readily from it is a function of the irritant. He observed that strong irritants, as *Staphylococcus aureus* or aleuronat, produce a prompt fixation which may occur as early as thirty minutes to one hour after the introduction of the irritant.

TABLE 8.—ADAPTATIONS OF MICRO-CHEMICAL METHODS FOR USE WITH THE PHOTOELECTRIC COLORIMETER

Substance	Changes necessary	Light filter
Urea . .	Use a blank of 7 ml. H_2O + 1 ml. Nessler's for 0 adjustment of galvanometer	Chance OB. 2 or Ilford minus red.
Uric Acid .	Dilute tests and standard to 5 ml. with water (very high ones to 10)	Chance OR. 2 red, or Ilford tri-colour red.
Creatinine .	Dilute tests and standard to 5 ml. with water. Use a blank consisting of water + 0 5 ml. of alkaline picrate	Chance OB. 2 or Ilford minus red.
Phosphate .	No change	Chance OR. 2 red, or Ilford tri-colour red.
Cholesterol .	No change	Chance OR. 2 red or Ilford tri-colour red.
Glucose . .	Colorimetric method. Dilute with water to 10 ml., or 15 or 20 ml. for very high sugars. Use a blank for 0 adjustment	Chance OR. 2 or Ilford tricolour red.
Sulphonamides	No change	Chance OGr. 1 green or Ilford tricolour green.
Proteins and N.P.N. .	Use a blank consisting of 0 2 ml. 50 % H_2SO_4, 5 ml. water and 3 ml. Nessler's	Chance OB. 2 or Ilford minus red.
Bilirubin . .	Use 7 ml. of 85 % alcohol instead of 3 ml. abs. alcohol. This gives final vol. of 8 ml. instead of 4 ml. and standard is now equal to 3·2 mg. bilirubin per 100 ml.	Chance OGr. 1 green or Ilford tricolour green.
Sodium . .	1 ml. of coloured solution diluted to 10 ml. with 0 5 % acetic acid	Chance OB. 2 or Ilford minus red.
Phosphatase .	Dilute to 10 ml. with water. (High phosphatase further diluted)	Chance OR. 2 red, or Ilford tri-colour red.
Potassium .	No change	Chance OR. 2 red or Ilford tri-colour red.
Haemoglobin .	All methods described	Chance OGr. 1 green or Ilford tricolour green.

a subsequent section (Chapter XIII). He pointed out at the time that doubtless some, but not *all* of the microorganisms disseminate at once from the site of any tissue inoculation (cf. Fig. 34). In a non-specific inflammatory reaction there is nothing to restrict the dissemination of some of the bacteria immediately after their inoculation and prior to the development of lymphatic blockade. The studies of Menkin (1929), of Hudack and McMaster (1932), and of Lurie (1936) indicated that there seems to be an increase or at least an unimpeded lymphatic drainage in the very initial stage of an acute inflammation. At the same time, it is clear that the rapidity and intensity with which the inflammatory reaction develops will determine the ultimate number of microorganisms capable of penetrating to the circulating blood stream. There is, therefore, little doubt that without the developing allergic inflammation in tuberculosis the spread of the reinfecting bacilli would be unobstructed. The number of bacilli disseminating in the earlier stage of reinfection prior to the development of a sufficiently intense allergic inflammation are probably few, and therefore can be disposed of adequately in the tributary lymph nodes, i.e. provided their virulence is not too great.

Lurie (1939) in a recent study on the rôle of extracellular factors in the mechanism of immunity in tuberculosis has further substantiated this point of view. He has demonstrated that in the reinfected guinea pig the lymphatics adjoining the site of reinfection become thrombosed, whereas in the rabbit whose tissues are less injured by the bacilli the lymphatics remain unoccluded. In the guinea pig, moreover, in contrast to the rabbit, the fibrinous network in the inflamed area forms a closely knit, sieve-like structure. These findings essentially confirm the earlier observations of the writer (1931*a*) on the mechanism of fixation. This question will be considered in greater detail in a subsequent chapter. Suffice it to say that Lurie's observations add further support to the view that the allergic inflammation in tuberculosis doubtless plays a significant rôle in preventing the dissemination of the bacilli of reinfection.

light, provided the light is invariably of the same intensity and wave-length. With any given coloured solution and the proper light filter, therefore, the same difference in galvanometer deflection between water and coloured solution should be obtained. This is the case for any one instrument; different instruments may differ sufficiently in details of construction to give slightly different deflections with the same solution. By calibrating an instrument against a series of standards a factor may be derived or a graph constructed for the calculation of the concentrations of subsequent test solutions of unknown strength. But it must be recognized that the accuracy obtained in this way may not be as great as that which will result from the simultaneous comparison of a standard with the test solution. The reagents used in any procedure may vary slightly from day to day—e.g., by deterioration—and as a consequence the colour produced on one occasion may not be absolutely identical with that produced on another. Moreover, the selenium cell may suffer small alterations in its sensitivity and as a consequence the deflection resulting from any given incident light may vary slightly. However, the accuracy obtainable may be sufficient for many routine examinations. If it is desired to use the colorimeter in this way, a separate set of standard graphs or factors should be established for each instrument.

PERMANENT STANDARDS FOR THE TURBIDOMETRIC ESTIMATION OF PROTEIN

The turbidometric procedure of Kingsbury *et al.* (1926) can be applied to urine, C.S.F. and other body fluids, and has been in use, with modifications in the apparatus required, in the laboratories of the British Postgraduate Medical School for some time. The procedure is very simple, and can be used with equal success by laboratory workers and general practitioners.

The principle of the method consists in comparing the turbidity in a set of permanent standards with that produced when a standard amount of sulphosalicylic acid is added to a measured amount of the albuminous fluid. The

ignored. This interpretation is considered by Follis not to be a valid one (1938). It is conceivable that the experimental elimination of hypersensitiveness does not preclude the possible importance of allergic manifestations when all the other factors concerned in the mechanism of immunity are evaluated. At present we are unfortunately still ignorant as to whether or not other compensatory factors are brought into play to promote resistance during the process of desensitization.

In general it may be said that the extensive studies of Rich and of his collaborators have thrown some doubt on the widely accepted view that allergy is the essential mechanism of immunity in tuberculosis (Krause and Willis, 1924; Zinsser, H., Ward, H. K., and Jennings, F. B., Jr., 1925). The basic observations of Lurie have indicated that immunity in tuberculosis is a "function of the increased capacity of the mononuclear phagocytes to digest tubercle bacilli" (1933, 1936). In a more recent study, Lurie (1939*a*) has succeeded in demonstrating that mononuclears derived from tuberculous animals exhibit greater *in vitro* phagocytic ability for tubercle bacilli than mononuclears obtained from normal rabbits or guinea pigs. This enhancement in phagocytic capacity on the part of cells from vaccinated animals is doubtless of great significance in an understanding of the mechanism of immunity in tuberculosis. The earlier work of this investigator has furthermore suggested the presence of an additional differential local organ immunity. In the spleen, liver, and bone marrow of an infected rabbit, tubercle bacilli are destroyed more rapidly than in the lungs and kidneys (Lurie, 1928). In the allergic animal these same differences are essentially maintained, producing merely an enhanced destruction of the reinfecting bacilli (Lurie, 1929). The studies of Lurie support the view that besides allergy there are other factors involved in the mechanism of immunity in tuberculosis. Genetic factors and racial differences in the human disease are probably additional important elements (Opie, McPhedran, and Putnam, 1936;

trations. The following mixtures of gelatin and formazin-gelatin suspension are made in small tubes of uniform bore (7·5 by 1 cm.)

TABLE 9

Tube	ml of gelatin	ml of gelatin-formazin	Value in terms of albumin per 100 ml
1	3·6	0·4	10
2	3·2	0 8	20
3	2 8	1·2	30
4	2·4	1·6	40
5	2 0	2·0	50
6	1·6	2·4	60
7	1 2	2 8	70
8	0 8	3 2	80
9	0·4	3 6	90
10	0	4 0	100

When cold, the tubes are stoppered with corks cut level with the top of the tube. The stoppered ends are then dipped in molten paraffin wax and allowed to cool. The standards may be checked against serum solutions standardized by nitrogen determinations. They should be mounted in a wooden rack painted black. Comparison is best made by viewing them against a strip of white cardboard with a transverse black line fastened to the rack.

Arrangements have been made with Messrs. Gallenkamp and Co., London, to manufacture these standards. Sample sets have been checked against those prepared in this laboratory and against albumin solutions of known strength.

COLORIMETRIC STANDARDS FOR EMERGENCY ESTIMATIONS OF CERTAIN CONSTITUENTS OF BLOOD AND C.S.F.

The determination of urea in blood is most easily carried out by conversion of the urea into ammonia by urease, followed by direct Nesslerization; that of non-protein nitrogen (N.P.N.) and plasma proteins by suitable digestion with sulphuric acid, whereby the whole of the contained

the inflammatory reaction play a definite rôle in the mechanism of immunity by retarding the dissemination of bacteria? The various types of evidence already cited as well as others to be discussed later, confirm the viewpoint that an acute *inflammation, either of specific origin or caused by a non-*specific irritant, definitely delays the spread of injurious substances from the site of inflammation. The principle of the Koch phenomenon applied to human tuberculosis is probably still the best available explanation to distinguish adult from childhood tuberculosis. The various studies of Küss, Ghon, and Opie indicate, in the words of the latter (1936) that: "The peculiar characteristics of adult or reinfection type of pulmonary tuberculosis are referable to allergy induced by a first infection." The recent experimental work of Freund and Angevine (1938) essentially confirms the earlier work of Willis (1925) on the failure of tubercle bacilli to disseminate freely from the skin of immunized animals. The retardation was found to be due to local fixation and not to destruction of the bacilli. The available facts therefore strongly suggest that the allergic inflammation is quite likely one factor among several others which is involved in the manifestation of immunity in tuberculosis. Its importance lies in the tendency of the acute inflammation of hypersensitiveness to restrain the free penetration of reinfecting bacilli. Unfortunately, at present there seems to be no available method of evaluating in a quantitative manner the relative significance of each of the various factors involved in the mechanism of immunity in tuberculosis (Menkin, 1938).

Fixation of Pyogenic Bacteria in Immune Animals

Wadsworth (1904) injected virulent pneumococci into the trachea of normal animals and thereby caused a fatal bacteremia which in some cases was associated with bronchopneumonia. When immunized animals were treated in the same manner, there was a diffuse exudation comparable with lobar pneumonia, but no invasion of the blood stream. Cecil and Blake (1920) also found that the incidence of invasion of

TABLE 10.—COMPOSITION OF PERMANENT STANDARDS

Standard value (N equivalent) mg.	ml. of Ferric Chloride Solution *	ml of Cobalt Chloride Solution *	0·1 N-HCl
0·015	1·1	0·15	0·1 N-HCl added to make the volume 8 ml.
0·02	1·4	0·27	
0·025	1·75	0·40	
0·03	2·1	0·55	
0·035	2·4	0·68	
0·04	2·75	0·80	
0·045	3·1	0·93	
0·05	3·4	1·05	
0·055	3·75	1·18	
0·06	4·1	1·30	
0·07	4·7	1·55	
0·08	5·4	1·80	
0·09	5·9	2·10	

* Ferric chloride solution: 10 per cent. (w./v.) $FeCl_3.6H_2O$ in 0·1 N-hydrochloric acid—i.e., 10 g. of the salt dissolved in enough 0·1 N-hydrochloric acid to make a final volume of 100 ml. Cobalt chloride solution: 10 per cent. (w./v.) $CoCl_2.6H_2O$ in 0·1 N-hydrochloric acid.

values are spaced more closely in order to allow of more accurate estimation of plasma proteins. For total proteins the increments of 0·005 mg. N correspond to 0·78 per cent. protein. By careful matching intermediate values can be assigned, and it should hence be possible to gauge the concentration of protein to within 0·4 per cent. On the same basis it should be possible to measure fibrin to within 0·02 per cent. and albumin to within 0·2 per cent. Since globulin is estimated by the difference between total protein and albumin the error may be additive, and at the most should be 0·6 per cent. For C.S.F. protein the increments of 0·005 mg. N correspond to 6 mg. per 100 ml. of protein, and half-increments to 3 mg.

An alternative rapid method for plasma and C.S.F. proteins, based on the xanthoproteic reaction, may also be used.

The colour of iodine in ether solution is very much like that of the Nessler-yellow. The estimation of sugar by a method in which the final determination is of liberated iodine should make it possible to estimate blood sugar by means of the permanent standards. The Schaffer-Hartman procedure was easily adapted to this purpose. The intensity of the

avirulent and virulent streptococci from the site of their inoculation to the regional lymph nodes. He found, after immunization associated with sensitization, that virulent streptococci were more readily localized in the skin and destroyed by phagocytosis. With a relatively avirulent strain of hemolytic streptococci he observed in sensitized animals a marked local inflammation. The microorganisms disseminate *from such an area to the tributary lymphatic nodes* in appreciably smaller number.

Rich and McKee (1932, 1934) have come to the conclusion that local immobilization of bacteria (Pneumococci, type I) in a reinfected immune animal is primarily due to the presence of agglutinating tissue antibodies which prevent the dissemination of the microorganisms. The localization is *immediate and precedes the development of fixation by* inflammation. According to Rich (1933), when the acute inflammatory reaction is finally established, it no doubt is of assistance in preventing the further spread of bacteria. The writer (1930*a*), on the basis of his earlier studies on the concentration of proteins in an inflamed area, agrees essentially with this viewpoint. The question is, however, raised whether the presence of part of the tissue agglutinins in an immune animal is not the result of accumulating antibodies from the circulation (see Chapter X). Inflammation, as has been pointed out (Menkin, 1933), may at certain initial stages reveal other manifestations besides leukocytic migration. The early increase in capillary permeability, although perhaps not detectable microscopically, is nevertheless as much a part of the inflammatory reaction as is the subsequent infiltration of leukocytes. Consequently the conspicuous concentration of antibodies in an area of reinfection does not necessarily imply a phenomenon independent of inflammation. The usual antibody content in a local area of an immune animal (Freund and Whitney, 1929) may thus be simply enhanced by a further accumulation of agglutinins through the damaged capillary wall. The prompt concentration of antibodies from the circulation in an area of specific

(mg. N) obtained on comparing with the standards is multiplied by 125—i.e., $\frac{100}{0\cdot005} \times \frac{6\cdot25}{1000}$ —to obtain the percentage of total protein.

Albumin (p. 36). The following are the calculations entailed:

(1) *By Difference with N.P.N.*:

$$\text{mg. albumin N} + \text{N.P.N.} = \text{Reading} \times 10{,}000 \left(\text{i.e., } \frac{100}{0\cdot01}\right)$$

$$\text{mg. albumin N} = (\text{Reading} \times 10{,}000) - \text{N.P.N.}$$

$$\text{percentage albumin} = \frac{\text{mg. albumin N} \times 6\cdot25}{1000}$$

(2) *By Precipitation of the Albumin*:

$$\text{percentage albumin} = \text{Reading} \times 62\cdot5 \left(\text{i.e., } \frac{100}{0\cdot01} \times \frac{6\cdot25}{1000}\right)$$

Globulin. Total protein minus albumin.

Fibrin. The value obtained for the colour on matching with the standards is multiplied by 6·25—i.e., $\frac{100}{0\cdot1} \times \frac{6\cdot25}{1000}$; the result is the percentage of fibrin.

C.S.F. Protein. 0·1 ml. of C.S.F. is digested with 0·2 ml. of 50 per cent. sulphuric acid as in the method for plasma total protein. The value obtained on comparing with the standards is multiplied by 1000—i.e., $\frac{100}{0\cdot1}$ —to give the total mg. N per 100 ml. C.S.F. The N.P.N. of the C.S.F. is estimated by digestion and Nesslerization of 1 ml. of a 1 : 10 protein-free filtrate, equivalent to 0·1 ml. C.S.F. (0·5 ml. of C.S.F. + 3·5 ml. of water + 1 ml. of 25 per cent. trichloroacetic acid). The result is multiplied by 1,000 to give the N.P.N. (mg. per 100 ml. of C.S.F.). The N.P.N. is subtracted from the total N to give the mg. protein N in 100 ml. This figure is multiplied by 6·25 to give the mg. protein per 100 ml. C.S.F.

Estimation of Plasma and C.S.F. Protein by the Xanthoproteic Reaction. When protein solutions are heated with

(Menkin 1938c) observed that the apparently normal skin of the abdomen may display an increased glycolysis when an acute cutaneous inflammation has been set up at some distance from the normal area. It is to be recalled that these workers brought forward evidence indicating that the severity of a lesion seems to be related to the intensity of glycolysis (1937). Biochemical observations of this sort suggest that perhaps a local cutaneous inflammatory reaction may be of significance in altering the normal metabolism of a considerable area of what is otherwise considered uninvolved skin. This possibility may have to be taken into account when studying absorption of material from the site of skin inoculation.

Summary. Specific inflammation in the immune body is exemplified either as an allergic or as an anaphylactic inflammatory reaction.

A typical example of an allergic inflammation is seen in tuberculosis. The reinjection of tubercle bacilli or of tuberculo-protein in a tuberculous animal induces an acute local exudative inflammation with failure to involve the satellite lymph nodes. This well-known reaction is termed the Koch phenomenon. The tuberculin reaction is essentially a manifestation of the same phenomenon elicited with tuberculo-protein. In contrast to the Arthus phenomenon (anaphylactic inflammation) the tuberculin reaction is not passively transferable to a normal animal by the injection of serum from a tuberculous animal. The tissue culture studies of Rich and Lewis (1928) suggest that in tuberculous tissue the specific antibody might be directly attached to cells or at least in close association with them. Rich and his collaborators maintain that acquired immunity is in no way dependent on the development of allergic inflammation. A different view is upheld by a number of other investigators (Willis, Lurie, Freund and Angevine, Opie, and Menkin) whose studies either on tuberculosis or on the general mechanisms of inflammation indicate that the allergic inflammatory reaction probably plays an important rôle in immunity by re-

and of normal H_2SO_4 are added. Two ml. of ether * are now added and the mixture well shaken. The iodine will be seen to have gone into the ether layer. The colour is compared with the standards, and the sugar value derived from Table 12.

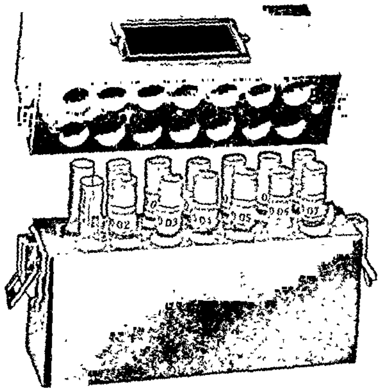


FIG. 16.—Permanent artificial colour standards for the emergency estimation of blood and C.S F. constituents.

If the colour obtained is less than the 0·02 standard the test is repeated with 1 ml. of blood filtrate plus 1 ml. of water, and the result multiplied by 2. *C.S.F. sugar* may be estimated by the same procedure.

* It is essential that the ether be peroxide-free; otherwise excess iodine may be liberated from the potassium iodide. The ether may be tested by adding 2 ml. to a mixture of 2 ml. of water, 0·5 ml. of potassium iodide, and 0·5 ml. of sulphuric acid. If on shaking the mixture no perceptible yellow colour is present in the upper layer the ether is satisfactory for the test. In practice only a few samples of ether which had been stored in glass for a long time have proved unsatisfactory.

intradermal injection immediately enters these vessels. They found that lymphatic capillaries in regions injured by heating, ultra-violet light, or by intradermal injection of bacterial toxins are definitely more permeable than under ordinary circumstances. Diffusible dyes injected into such areas escape with extreme rapidity in striking contrast to their behavior when placed in contact with normal cutaneous regions. In brief, they found that if the inflammatory process has not progressed to a purulent or to a necrotic stage there is a more rapid turnover of such material from the inflamed area.

They also studied the reaction of lymphatic capillaries in the ears of mice immediately after minor injuries, such as are produced by incisions or mild burns (1934). Whereas small blood vessels tend to close after an incision, the lymphatics remain open for as long as forty-eight hours. Consequently materials introduced into such wounds pass directly into lymphatics through their gaping ends. This may explain the dissemination from cutaneous abrasions of infectious agents through lymphatics. As in the case of vascular capillaries, the lymphatic wall about an area of injury is rendered abnormally permeable. It is quite possible that this increased permeability is likewise referable to the liberation of leukotaxine (Menkin, 1938*c*). Twenty-four to forty-eight hours later McMaster and Hudack found lymphatics less permeable than usual. Probably by that time the inflammatory reaction had reached a sufficient degree of intensity to favor plugging of lymphatics. It is conceivable that the observations of Falchetti and Carlinfanti (1933) on the enhanced development of anaphylactic shock, when a homologous antigen is injected into an inflamed area induced by B.C.G., can be explained by the more rapid diffusion of the foreign protein from a site of relatively mild injury.

The observations of Hudack and McMaster are of great significance, for they clearly demonstrate that, in the initial stage of an acute inflammation, the lymphatic vessels undergo changes similar to those encountered in capillaries,

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6

prompt accumulation of foreign proteins from the circulation in inflamed foci. Fox (1936) has also recently obtained evidence suggesting that antibodies tend to localize in such areas. In view of these facts, it is therefore reasonable to suppose that in an allergic or anaphylactic inflammation the mechanism of fixation is probably reinforced by the presence of specific antibodies (see Chapter XI). This view was foreshadowed by Opie (1924) in his investigations on the mechanism of the Arthus phenomenon, and it was later developed further by Rich and McKee (1934) in their interesting studies on the immune animal. The recent interpretation by Hadfield and Garrod (1938) that the studies of Rich and those of the writer represent divergent views concerning the mechanism of localization in inflammation is open to question. Rich has studied the reactions in immunized animals, whereas the writer has devoted his attention primarily to the mechanism concerned with the non-specific type of inflammation. The findings on the immune rabbit do not conflict *with the results obtained on the non-immunized animal.* The presence of antibodies in sensitized tissue and their accumulation in part from the circulation merely reinforces the basic non-specific (cf. Chapter XI) mechanism of fixation as manifested in the non-immune animal.

Opie (1936) believes that it is difficult to estimate the relative importance of leukocytes, antibodies, and of the inflammatory reaction itself in local fixation. Careful analyses, however, of the mechanism involved in regard to the element of time, or what may be termed the threshold of fixation, supports the view that lymphatic blockage constitutes the basic reaction but that, as often maintained, it may be reinforced by various factors (Menkin, 1931*a*, 1932*a*, 1933, 1937*a*). Whereas the latter, in turn, may be quite significant, they are nevertheless considered secondary for the simple reason that they do not have to be present for the reaction of fixation to take place. As has often been pointed out by the writer, his observations indicate that with some powerful irritants, which cause considerable necrosis (for instance,

from which an injected vital stain (trypan blue) failed to reach the tributary lymph nodes.

2. It was reasoned that, if mechanical obstruction plays an important rôle in fixation, then substances which are un-

FIG. 35. The mesh of fibrin at the periphery of a zone of infiltration (From Menkin, *Arch Path.*, 1931, *12*, 802.)

able to disseminate from an acutely inflamed area should, for the same reason, fail to enter it when injected at the periphery of the area. This is precisely what was found to occur when trypan blue was injected at the periphery of an

It is evident that whereas *B. prodigiosus* penetrated freely into the normal skin areas, considerably fewer organisms entered the site of inflammation. Burrows has confirmed this experiment with India ink (1932).[1] Studying the living frog under the microscope, the writer has followed the diffusion of a dye from its peripheral inoculation to the

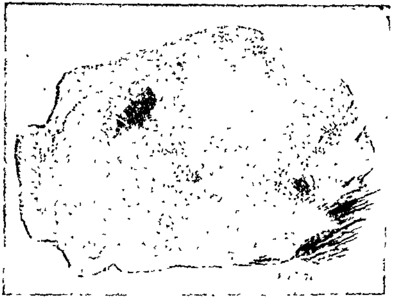


FIG. 37. The failure of trypan blue to penetrate a site of inflammation when injected at its periphery: 0.1 cc. of trypan blue was injected into each of several areas surrounding an inflamed area. About one and a half hours later the dye had diffused into the surrounding normal tissues, but had failed to penetrate the site of inflammation. (From Menkin, *Arch. Path*, 1931, *12*, 26)

boundary of an inflamed area where its further progress was blocked by the presence of a thrombus occluding a lymphatic channel (1931*d*).

3. A third type of evidence which reinforces the hypothesis was obtained by taking advantage of the fact that concentrated urea dissolves fibrin. When this solvent was injected with or immediately after the irritant, obstructing thrombi

[1] The recent studies of Avery and Rigdon (*Proc Exp. Biol. and Med.*, 1939, *42*, 67) indicate that a chronic inflammation induced by the preliminary injection of

fails to do so when introduced around an inflamed area consequent on the injection of distilled water and bacteria (*Staph. aureus*).

These three distinct types of evidence render it quite likely that the basic mechanism of fixation in inflammation is primarily referable to an obstructing barrier in the form of thrombi in lymphatics and of coagulated plasma in edematous tissue. There is as yet no precise information on the

FIG. 39. Retrosternal lymphatic vessel draining peritoneal cavity about seven hours after it had been treated with
by 10 cc. of distilled water. The lu
containing leukocytes in abundance.
toneal cavity failed to disseminate
X 145. (From Menkin, *J. Exp. Med*, 1932, *56*, 157.)

question of the relation of increased capillary passage to the lymph flow from an inflamed area. Pending such data, the finding of increased lymph flow from a main lymphatic trunk draining a large area of tissue injured unequally by the inflammatory irritant only indicates, as would be expected, an absolute increase in lymph flow. In such an experiment one cannot preclude the possibility of a relative decrease in the lymph output when the total amount of plasma which has

permeated through the capillaries is taken into consideration (Menkin, 1933*b*). Inflammatory edema may in part be an index of the inability of a partially impaired lymphatic circulation to take care of the excessive passage of fluid from the circulating blood into acutely inflamed tissue. In this connection the interesting observations of Drinker and Field are suggestive and instructive (1933). These investigators have succeeded in developing a conspicuous local edema or

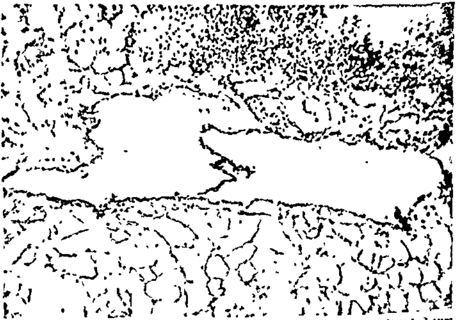


FIG. 41. Retrosternal lymphatic vessel draining peritoneal cavity six hours and thirty minutes a [illegible] in 50 per cent urea. [illegible] seminated freely to [illegible] inflammatory reaction. X 360. (From Menkin, *J. Exp Med*, 1932, 66, [illegible])

elephantiasis in the leg of dogs following obstruction of the lymphatic trunks.

Burrows (1932) recently reviewed the various factors involved in the mechanism of inflammatory retention. According to this author these may be briefly stated to be as follows: a, increased osmotic pressure; b, stasis and clotting of lymph; c, phagocytosis; d, agglutination of bacteria *in vivo*; and e, the electrostatic conditions in the tissues as well

as the electrical charges on the particles which are retained in an area of acute inflammation. Schade and Menschel (1923) have found that in inflamed areas, especially those with suppuration, the accumulation of products of tissue disintegration may become so great that the osmotic pressure is raised to as high as eleven atmospheres, with a simultaneous marked increase in hydrogen ion concentration. They believe that inflammatory edema has primarily an osmotic origin. For this reason it seems reasonable enough to suppose that the increase in osmotic pressure at the site of inflammation may be a factor in influencing the flow of fluids and perhaps indirectly any contained substance. It is, however, doubtful whether this factor plays a significant rôle in fixation. In the first place, determination of the osmotic pressure of exudates obtained in the *early* phase of an acute inflammatory reaction usually yields a value not markedly different from that of blood serum (Menkin and Warner, unpublished). Yet the fixation of various substances at such an early period in the development of an acute inflammation can be readily demonstrated. Furthermore, the fact that substances injected at the periphery of an acutely inflamed area fail to penetrate into it (Menkin, 1931*a*) seems to invalidate the view that retention is primarily referable to an increase in osmotic pressure within the injured tissue. As pointed out previously, phagocytosis and agglutination *in vivo* are doubtless important secondary factors. Fixation, however, can be readily demonstrated in the absence of these factors (Menkin, 1937*a*). When brought into play they are of considerable significance in reinforcing the basic mechanism of lymphatic blockade. The suggestion of differences in electric charges to account for fixation is an interesting view, which, however, seems as yet to have little experimental basis to substantiate it.

In brief, the evidence on hand which has received further support from the studies of Burrows (1932), of Clark and Clark (1937), and of Dennis and Berberian (1934), indicates that the basic mechanism of fixation in an acute non-

specific inflammation seems to be primarily referable to the establishment of an effective lymphatic blockade (Menkin, 1931*a*). These studies recently have again received confirmation in the hands of Lurie (1939). This worker has demonstrated that in the guinea pig the tubercle bacilli of reinfection are fixed at the portal of entry. This animal is likewise capable of localizing, at the site of reinfection, unrelated material such as trypan blue or agar particles. This is referable to a marked degree of local injury in the animal which favors occlusion of lymphatics and the deposition of a dense fibrinous network at the site of inflammation. Other elements, such as specific or non-specific precipitation, agglutination *in vivo*, size of particle or diffusibility, adsorption,[1] or phagocytosis, represent various secondary factors which, under special circumstances or at some phase of the inflammatory reaction, may be of great significance in reinforcing the primary and basic mechanism of fixation.

Experiments described in previous chapters (IX and XI) present proof that various foreign substances including dyes, a metallic salt, foreign proteins, particulate matter, and bacteria, *injected into the site of an acute inflammation* are fixed *in situ* and fail to drain readily into the tributary lymphatic vessels. These same substances, as pointed out in Chapter X, injected intravenously, accumulate rapidly in inflamed areas. This accumulation is partly associated with increased permeability of the capillaries, but is also the result of the inability of these substances to escape from the site of inflammation, owing to the presence of a fine network of fibrin and of thrombosed lymphatics. With some irritants the mechanism of fixation may occur extremely early in the inflammatory process. In the writer's earlier experiments (1929) fixation of a dye at the site of inflammation could be demonstrated by study of the regional lymphatics as early

[1] It is interesting to note that trypan blue poured over a clot in a test tube not only fails to permeate the interior of the coagulum, but the dye shows barely any trace of having been adsorbed on the fibrinous material This observation is perhaps of some interest in view of recent comments by Rich (1936), who has raised the question of the possible adsorptive capacity by fibrin to account for fixation at the site of inflammation.

as thirty minutes after the injection of the inflammatory irritant. This would substantiate the view, as pointed out in a foregoing section (Chapter IV), that the earliest change in inflammation is an increase in the permeability of the capillaries which permits the passage of fibrinogen from the plasma into the tissue spaces. The rapid formation of a network of fibrin and of thrombi in lymphatics at the site of inflammation circumscribes the irritating substance and thus prevents its passage into the blood stream. Early fixation thus serves as the forerunner of the events whereby the disposal of the foreign material occurs at the site of inflammation. By circumscribing the irritant it gives an interval of time for the leukocytes to assemble for the purpose of phagocytosis. The initial barrier caused by thrombosed lymphatics and coagulated serum in the tissue spaces of the inflamed area thus plays a definite rôle in immunity by protecting the essential organs at the expense of local injury.

Summary. Minor tissue injury favors the dissemination of injected foreign materials into the tributary lymphatic channels. On the other hand, the "walling off" of an acutely inflamed area seems to be due to an enhanced passage of fibrinogen through the more permeable capillary wall. The mechanism of fixation, as indicated by three distinct types of evidence, is primarily referable to the formation of a fibrinous network and of thrombi occluding the lumina of draining lymphatics. This favors the development of lymphatic blockade in acute inflammation. Various secondary factors, such as, for instance, the presence of immune bodies in anaphylactic or allergic inflammation, may reinforce the basic mechanism. The early occurrence of fixation in a severely injured area plays a definite rôle in immunity, for it allows an interval in which the relatively sluggish leukocytes may assemble for the purpose of phagocytosis.

CHAPTER XIII

INFLAMMATION AND BACTERIAL INVASIVENESS IN RELATION TO RESISTANCE

Mechanism of Bacterial Invasiveness

The observations on the mechanism of fixation have offered a means of studying a factor that may account in large part for the differences in the invasive ability of various pyogenic organisms (Menkin, 1933, 1935).

The intensity and rapidity with which an inflammatory irritant is circumscribed in a tissue area varies with the type of irritant. The speed with which an irritant causes a region to be walled off by thrombosed lymphatics, or a fibrinous network, or by both, is necessarily an important index in *determining its ability to disseminate ultimately into the circulating blood.* This holds true if the irritant can readily drain through lymphatic vessels from the site of inoculation. That bacteria disseminate from the site of inoculation through lymphatics has frequently been demonstrated (Noetzel, 1906; Wells and Johnstone, 1907; Menkin, 1931*b*). It follows from this that, if bacteria are employed as inflammatory irritants, some information may be obtained concerning the invasive capacities of different microorganisms. This would depend on the type of inflammatory reaction which a given microorganism induces in the host. Pathologists are familiar with the localizing tendency of staphylococci in contrast to the invasive property of the streptococci. Can the behaviors of various pyogenic organisms in tissues be related, in part at least, to their respective properties of creating an early inflammatory reaction at the site of their inoculation which would tend to prevent by the mechanism mentioned above, their dissemination to the tributary lymphatic nodes? When a vital dye is introduced into an area at

various intervals of time after bacterial inoculation of this site, the degree of walling off of the infected focus may be determined by the extent to which the dye can drain into the regional lymphatics.

TABLE XXII

RETENTION OF TRYPAN BLUE AT THE SITE OF STAPHYLOCOCCUS INFLAMMATION

RABBIT No	INTERVAL BETWEEN INJECTION OF IRRITANT AND THAT OF DYE	TOTAL DURATION OF INFLAMMATION	PRESENCE OF DYE ON INFLAMED SIDE		PRESENCE OF DYE ON NORMAL SIDE	
			Lymph of Efferent Lymphatic	Lymph Node	Lymph of Efferent Lymphatic	Lymph Node
	hrs.:min.	*hrs :min.*				
7-05	0.0	2 23	++	++	++	+++
7-07	0·30	1.52	+++	+++	+++	+++
10-26	1:03	3·33	Faint trace	Trace	+	+
10-26A	1:43	4.00	0	Faint trace	Trace	+
6-61	1.35	4·07	Faint trace	0	++	+
7-18	1·45	4 07		Trace		++
10-27	3·43	5:55	0	0	+	++
10-21	17:38	19 45	0	0	++	++
10-22	17:40	19·55	0	0	++	+
10-20	21:50	23.00	0	Trace		++
10-23	21:05	23:16	0	0	Trace	+++
10-24	20 57	23:28	0	0	++	++

From Menkin, *J. Exp. Med*, 1933, *57*, 977.

The observations represent a comparison of three pyogenic cocci, *Staphylococcus aureus*, *Streptococcus hemolyticus*, and Pneumococcus Type I, with regard to the rapidity of fixation of a vital dye at inflammatory foci set up by these respective organisms (Menkin, 1933). The significance of these experiments in reference to an understanding of the differences in the invasive properties of these three microorganisms will likewise be indicated. If, as shown in Table XXII, trypan blue was injected in both forelegs of a rabbit immediately after the inoculation of a broth suspension of *Staphylococcus aureus* in one of the limbs, the passage of the dye into the draining axillary lymphatics occurred in both the normal and inflamed side with about equal intensity. If, however, the dye was injected slightly over one hour after the bacterial

irritant, trypan blue reached the tributary lymph node or its efferent lymphatic vessel in greatly reduced quantities.

Histological studies were made of inflamed areas of about four to twenty-three hours duration at a time when trypan blue had been shown to be fixed at the site of staphylococcus inflammation. There was generally a moderate amount of edema as shown by the separation of tissues.

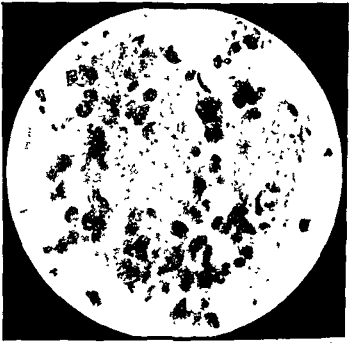


FIG. 42. Lymphatic vessels showing occlusion of their lumina by a delicate fibrinous reticulum. Taken from an area [illegible] of about four hours [illegible] mal circumstances X 895. (From Menkin, *J. Exp. Med*, 1933, *57*, 977)

Lymphatics were found with their lumina completely or in part occluded by a fibrinous reticulum (see Fig. 42, Rabbit 7–18, Table XXII). A meshwork of fibrin varying in extent was often seen at the periphery of a zone of polymorphonuclear infiltration. Clumps of staphylococci were frequently observed in close association with fibrinous strands.

The results obtained when *Staphylococcus aureus* is used

as an inflammatory irritant show that the inability of trypan blue to reach the regional lymphatics occurs as early as one hour after the onset of the inflammatory reaction, and that this fixation can be satisfactorily explained by the early formation of fibrin in tissue spaces and of thrombi in lymphatics at the site of inflammation.

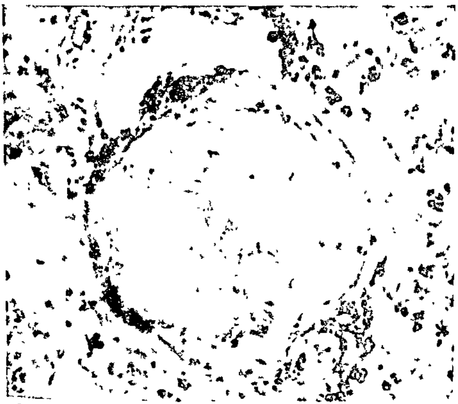


FIG 43 Thrombosed lymphatic vessel in a cutaneous area of inflammation induced by Pneumococcus Type I. The inflammatory reaction is of about twenty-three hours duration The dye injected into this inflamed area failed to reach the tributary lymphatics X 450 (From Menkin, *J Exp Med.*, 1933, *57*, 977)

Trypan blue introduced into an area of cutaneous inflammation induced by Pneumococcus Type I was retained in part when the dye was injected about six hours after the inoculation of the bacteria. The vital stain failed to reach the tributary lymphatic node if a period of over seventeen hours elapsed after the inoculation of the cocci.

Histological studies of areas inflamed for a period of about four hours revealed dilated and patent lymphatics with small infiltration of polymorphonuclear leukocytes in tissues distended with edema. When the inflammatory reaction is of longer duration (20 hours or over) the dye completely fails to reach the tributary lymphatics. At the same time, lymphatic vessels could be found that were completely occluded by dense thrombi (Fig. 43). In such sections the heavy cellular infiltration was conspicuous but the fixation was evidently associated primarily with the blockage of lymphatic vessels at the site of inflammation, inasmuch as a *fibrinous meshwork was seldom if ever observed in the tissue* spaces.

TABLE XXIII

RETENTION OF TRYPAN BLUE AT THE SITE OF STREPTOCOCCUS INFLAMMATION

Rabbit No	Interval between Injection of Irritant and That of Dye	Total Duration of Inflammation	Presence of Dye on Inflamed Side		Presence of Dye on Normal Side	
			Lymph of Afferent Lymphatic	Lymph Node	Lymph of Afferent Lymphatic	Lymph Node
	hrs min	*hrs min*				
6-59	1 44	4·06	+ + +	+ +	+ + +	+ +
6-71	6·07	10 08	+	+	+	+ +
10-10	17·05	19 20	+	+	+	+
10-16	17 50	20 14	+	+ +	+ +	+ to + +
10-12	17 50	20 40	+ +	+ +	+ +	+ +
10-15*	18 15	21 30		Faint trace		Trace
10-13	19 15	22·05	+ +	+ +	+ to + +	+
10-14†	19 10	22 15	Trace	+	Trace	Faint trace
10-11	23 45	26 15	+ + to + + +	+ + +	+ + +	+ + +
6-70	30·03	32 39	+	Trace	+	+ +
10-17A	45 30	47 33	0	0	+ +	+ +
10-18	45 25	47 40	0	0	+ + +	+ + +
6-58	45 21	47 41	+ + +	+ +	+ + +	+ + +
10-17	46 10	49 30	0	0	+ + +	+ + +
6-60	47 56	50 13	+ +	+ +	+ +	+ +
6-42	48 16	50 43	0	0	+ +	+
10-19	70 45	73 35	+	+	+ +	+ +
7-01	71 10	74 10	+	+	+ +	+ + +

* Smaller quantities of dye injected into each foreleg (amount—1.8 cc. into each foreleg).
† Smaller quantities of dye injected into each foreleg (amount—1 cc. into each foreleg).
From Menkin, *J. Exp. Med.*, 1933, 57, 977.

The results as concerns the retention of trypan blue in areas of inflammation induced by hemolytic streptococci (Strain S-23 of Lancefield) are shown in Table XXIII. This occurred at a considerably later period than with either

staphylococci or pneumococci. Definite evidence of fixation was not obtained until 45 hours after cutaneous inoculation with the microorganism.

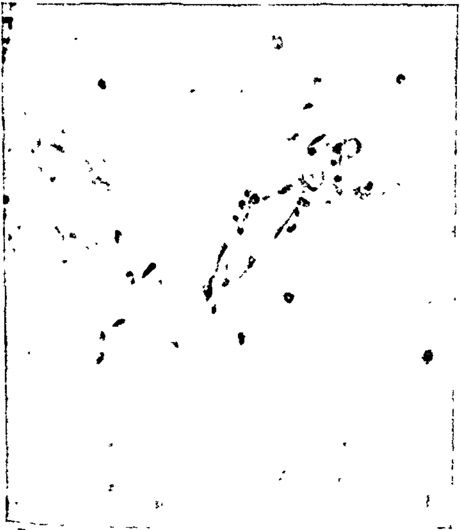


FIG 44. Lymphatic vessel in an area of inflammation induced by *Streptococcus hemolyticus*. The reaction is of about four hours duration. There is [illegible]

Histological studies revealed patent lymphatics at a time when trypan blue diffuses readily from a streptococcus inflammatory area (see Figs. 44 and 45 and Experiments 6–59

and 10-10). However, when the reaction has been going on for about two days and fixation of the dye has been demonstrated, one finds in addition to coagulated plasma and in-

FIG. 45. Lymphatic vessel in an area of streptococcus inflammation of about nineteen hours duration. The lur [illegible] into this area diffused freely to th [illegible] XXIII) Approximately X 450.

tense cellular infiltration, lymphatics occluded by dense leukocytic thrombi (see Fig. 46, Experiment 6-42).

The foregoing experimental results therefore clearly show that the rapidity with which an area of acute inflammation

becomes circumscribed so that foreign substances fail to spread readily from it is a function of the irritant. Staphylococci, by their high necrotizing power, cause the formation of thrombi in lymphatics and hence fixation within about one hour after their inoculation into normal cutaneous tissue.

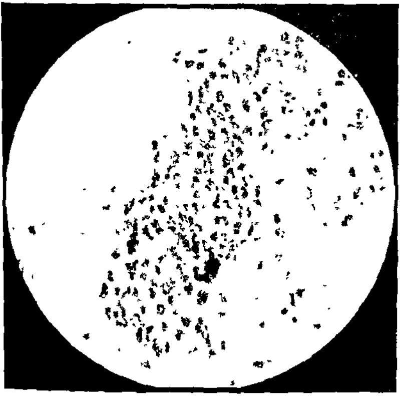


FIG. 46. Lymphatic vessel from an area of cutaneous inflammation induced by *Streptococcus hemolyticus* The inflammatory reaction is of over fifty hours duration. The lumen is occluded by a dense [illegible]

[illegible] 57, 977.)

The streptococci employed by the writer, on the other hand, accomplished this only about 45 hours later. The fact may account for the difference in invasive behavior of staphylococcus and streptococcus. It is conceivable, however, that there may also be other factors involved.

These observations present an interesting paradox (Men-

local disturbance in the intermediary carbohydrate metabolism (1937). Lesions induced by the *Staphylococcus aureus* manifest a considerably enhanced glycolysis in comparison to lesions of the same duration caused by hemolytic streptococci (1938c). The average concentration of glucose was found to be 62.3 mgm. per 100 grams of tissue in cutaneous areas previously inoculated with *Staphylococcus aureus* (Table XXIV). In skin areas of corresponding duration but injected with hemolytic streptococcus, the concentration of sugar averaged 129.7 mgm. per 100 grams of tissue. The level in normal skin was 149.9 mgm. These comparative figures indicate higher glycolytic activity in lesions induced by *Staphylococcus aureus*, and hence serve as further evidence for the view that the invasive behavior of microorganisms bears an inverse relation to the intensity of local injury.

TABLE XXV

RETENTION OF TRYPAN BLUE AT SITE OF INFLAMMATION INDUCED BY FILTRATE OF STAPHYLOCOCCUS CULTURE

Rabbit No.	Interval between Injection of Irritant and That of Dye	Total Duration of Inflammation	Presence of Dye on Inflamed Side		Presence of Dye on Normal Side	
			Lymph of Efferent Lymphatic	Lymph Node	Lymph of Efferent Lymphatic	Lymph Node
	hrs min	*hrs min*				
8–35	1 05	3 50	0	0	+	+
8–34	1 36	4 48	0	0	+	+
8–22	1 40	4 25	0	0	+	+
8–83	2 35	5 12	0	0	++	++
8–31	19 42	22 02	0	0	+	+
8–85	19 44	22 24	0	0	++	+++
98	20 20	22 50	0	0	Faint trace	+
9–74	22 28	24 39	+ to ++	+	+	++++
10–12	22 55	24 05	0	0	Trace to +	Trace to +

From Menkin, *Am J Med Sci.*, 1935, *190*, 583

2. The Berkefeld filtrates of several-day-old broth cultures of *S. aureus* induce an acute inflammatory reaction in the sk'n of rabbits which promptly causes retention of trypan blue (Table XXV, Fig. 47). The reaction is essentially indistinguishable from that obtained when the microorganisms alone are injected (Menkin, 1935). Heating the filtrate

to 58° C. for about one hour destroys the principle responsible for the reaction (Table XXVI, Fig. 48). The filtrate of a 24-hour culture is ineffective, whereas the one-day-old culture of the organisms produces a perfect walling-off of the inflamed area. This indicates that the *S. aureus* owes its

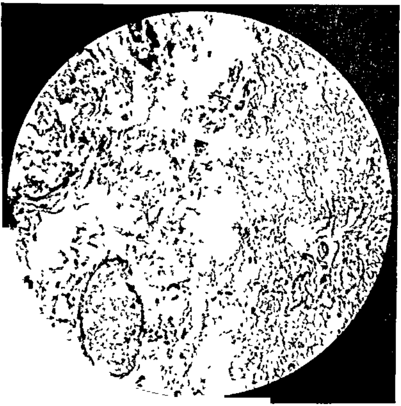


Fig. 47. An inflamed area induced by the intracutaneous inoculation of about 1.5 cc of the Berkefeld filtrate of a several-day-old culture of *S. aureus* (see Rabbit 8–85, Table XXV). The inflamed area is of over twenty-two hours duration Trypan blue injected into this area failed to reach the tributary lymphatics Note both the fibrinous thrombus occluding the lymphatic lumen, and the coagulated plasma in the tissue spaces (From Menkin, *Am J. Med Sci.*, 1935, *190*, 583.)

necrotizing activity not merely to its presence *per se* but in addition, also to its ability to release a powerful exotoxin-like product. This soluble, toxic, thermolabile material is capable of inducing sufficient capillary and lymphatic damage to produce a shunting off, so to speak, of the inflamed

area. There is some evidence that this material is identical with or at least resembles in many respects the staphylococ-

FIG. 48. A lymphatic lumen in an area of inflammation induced by the inoculation of inactivated *S. aureus* filtrate (This was accomplished by heating the filtrate at 58° C. for about one hour; see Exp. 5, Table XXVI.) The inflammation was of more than twenty-seven hours duration. The lumen is unoccluded. Trypan blue drained readily from this area to the tributary lymphatics (From Menkin, *Am J. Med Sci*, 1935, *190*, 583)

cus leucocidin of Denys and Van de Velde (1895). The filtrates of hemolytic streptococci and of Type I Pneumococcus are utterly unable to induce any such response. The dye

penetrates from such inflamed areas to the regional lymphatics even as late as 50 hours following the inoculation of streptococcal filtrates. The filtrates of these latter microorganisms are evidently entirely innocuous.

TABLE XXVI

Retention of Trypan Blue at Site of Inflammation Induced by Heat-Inactivated Filtrate of Staphylococcus Culture

Exp No.	Type of Staphylococcus Filtrate	Interval between Injection of Irritant and That of Dye	Total Duration of Inflammation	Presence of Dye on Inflamed Side		Presence of Dye on Normal Side	
				Lymph of Efferent Lymphatic	Lymph Node	Lymph of Efferent Lymphatic	Lymph Node
		hrs. min.	*hrs. min*				
1	Inactivated	17 50	20 55	+++	++	++	+++
	Normal	17 45	21 00	+	+	++++	+++
2	Inactivated	18 05	20 20	++	++	++	+
	Normal	17 55	20 20	Trace	0	+	++
3	Inactivated	20 10	22 25	++	+	++	+
	Normal	20 00	22 20	Faint trace	0	+	+
4	Inactivated	20 40	23 10	++	++	++	+
	Normal	20 33	23 05	Trace	Faint trace	+	+
5	Inactivated	25 25	27 10	+++	++	+++	++
	Normal	25 43	27 35	+	+	++	+++

From Menkin, *Am J Med Sci*, 1935, *190*, 583

3. A number of authors, including Much (1908), Gratia (1920) and Gengou (1933) have shown that the *Staphylococcus aureus* and its exotoxin are capable of causing oxalated blood to clot. According to Gratia this is due to a thermostable substance which he calls *staphylocoagulase*. Menkin and Walston (1935) have succeeded in separating *staphylocoagulase* from the fraction that induces inflammatory fixation. This was attained by treating the filtrate of a culture of microorganisms with glacial acetic acid. They found that the "clotting principle" elaborated by staphylococci, when injected intracutaneously, fails to exert a sufficiently powerful local reaction to obstruct lymphatic drainage. Trypan blue diffuses readily from the prepared site of cutaneous inoculation and the tributary lymphatics are found unoccluded. *Staphylocoagulase* evidently plays no rôle in inducing

an early lymphatic blockade. The latter is evidently referable to the powerfully necrotizing action *per se* of the staphylococcus microorganism and of its soluble toxin.

Invasiveness, Virulence, and Resistance

For the sake of clarity, the terms virulence, invasiveness, and resistance, as used by the writer, may be defined as follows:

Virulence represents the capacity of a pathogenic microorganism to produce a generalized deleterious effect when inoculated in a given host. The maximum effect of the damage is obtained with the death of the animal. The rate at which this end-point is reached serves as an index of the resistance of the host to a given dose of the infecting microorganism.

Invasiveness, on the other hand, represents the capacity of a microorganism to disseminate to the circulating blood from its point of cutaneous inoculation. The extent of invasiveness is evidently largely regulated by the intensity of the local inflammatory reaction induced in the host by the pathogenic organism.

As defined above, therefore, virulence refers to the ability of the microorganism *per se* to induce in the host generalized damage; whereas invasiveness is associated with the degree of localized inflammatory reaction developed in the host owing to the presence of the pathogenic microorganism.

Studies by the writer (1936c) clearly indicated that the local inflammatory reaction, by determining the degree of invasiveness, plays an important rôle in immunity. The resistance of the host infected with a virulent microorganism represents the resultant of a number of factors, including among others, the invasiveness and the virulence of the bacteria. The invasive property of a microorganism has in the past been taken as a criterion of its virulence and vice versa (Zinsser, 1931). Some bacteriologists, however, recognize now that virulence and invasiveness are different properties which are not to be confused and the terms are therefore not to be

employed synonymously (Topley, 1933; Zinsser, Enders, and Fothergill, 1939).

TABLE XXVII

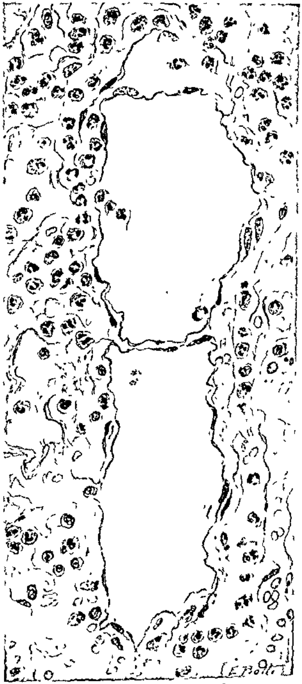
EFFECT OF A SUPERIMPOSED INFLAMMATORY IRRITANT ON THE SURVIVAL TIME OF RABBITS INFECTED WITH VIRULENT PNEUMOCOCCI

CONTROL ANIMALS				EXPERIMENTAL ANIMALS			
Rabbit No	Quantity of Pneumococcus Culture Inoculated	Quantity of Broth or Saline Injected	Survival Time	Rabbit No.	Quantity of Pneumococcus Culture Inoculated	Quantity of Staphylococcus Culture or Chemical Irritant Injected	Survival Time
	cc	*cc*	*days*		*cc*	*cc*	*days*
10-68	0 1	1 5 Broth	3	10-69	0 1	1 5 Staph	9
11-08	0 2	1 0 Broth	1	11-21*	0 2	1 0 Staph	4
10-54	0 2	3 0 Saline	1	10-53	0 2	3 0 Aleuronat	3
10-94	0 2	5 0 Saline	3	11-29	0 2	5 0 Aleuronat	5
10-66	0 25	3 0 Saline	4	10-64	0 25	3 0 Aleuronat	5
11-24	0 3	0 5 Water	2	11-28	0 3	0 5 Turpentine	11
10-78	0 3	1 5 Broth	5	10-77*	0 3	1 5 Staph	64 (Alive and well)
11-12	0 4	1 5 Broth	2	11-13*	0 4	1 5 Staph	3
11-99	0 5	1 5 Broth	2	12-00	0 5	1 5 Staph	3
11-02	0 5	1 5 Broth	3	11-01	0 5	1 5 Staph	4
11-97	0 5	1 5 Broth	4	10-86	0 5	1 5 Staph	9
10-61	0 5	1 5 Broth	3	11-98	0 5	1 5 Staph	34 (Alive and well—killed subsequently)

* With the exception of these rabbits, all animals were inoculated with the same strain of *Staphylococcus aureus*

From Menkin, *J Infect Dis*, 1936, *58*, 81

The factor of invasiveness may be studied as a separate variable by a number of experimental procedures. For instance, this was accomplished by interfering with the dissemination of a given virulent microorganism (Type III Pneumococcus) from its site of cutaneous inoculation (Menkin, 1936c). This end was attained by the superimposed injection of an inflammatory irritant that causes prompt "walling-off" (e.g. *Staphylococcus aureus*, aleuronat, or turpentine). Such manipulation, as shown in Table XXVII, delayed the dissemination of the virulent microorganism, and even, in some cases, apparently allowed sufficient time for the local inflammatory reaction to dispose of it in large part. In this way, the full effect of virulence, as manifested by massive blood stream invasion and subsequent death, could be re-



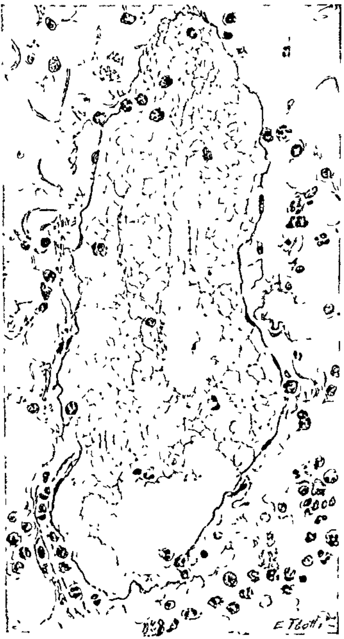


Fig. 50. [illegible]
induced [illegible]
lation. [illegible]
into the [illegible]
(From Menkin, *J. Infect. Dis.*, 1936, *58*, 81)

tarded and in a few cases aborted. The localizing tendency on the part of the cocci in experimental animals was found referable to the establishment of an effective lymphatic blockade. This was demonstrated both by the inability of an indicator dye, e.g. trypan blue, to reach the tributary lymphatic vessels and by histological examination of the inflamed areas (see Figs. 49 and 50). Nevertheless, there was no indication from these observations that the virulence or the toxic effect *per se* of the pathogenic microorganism had been in any way altered by delaying its invasiveness. For this reason, it is doubtful whether observations pertaining to virulence and resistance can be adequately utilized in drawing inferences concerning the function of an inflammatory reaction as a regulator of dissemination. Clark (1929) has shown that "preparing" a pleural cavity by preliminary introduction of an inflammatory irritant does not protect rabbits inoculated subsequently into that cavity with virulent Pneumococci Type I. Observations similar in nature do not indicate, as has apparently been assumed by some writers (Cannon and Hartley, 1938; Rich and McKee, 1934), that the acute inflammatory reaction has failed to prevent the *spread of virulent pneumococci. Fixation is not to be* measured by the resultant of a number of factors which collectively may perhaps be appropriately termed resistance. As shown by the writer (1931*b*), invasiveness or dissemina*tion is measured as a quantitative factor and therefore* even if but a few extremely virulent microorganisms can escape into the circulation through the mechanical barrier induced in an area of inflammation, their virulence may prove of suffi*cient magnitude to overcome the resistance of* the animal.[1]

[1] The complexity of factors that may be involved in evaluating resistance is exemplified in the various studies of different workers (e g Hughes, W. H, *J Path. and Bact*, 1937, *45*, 377, 1939, *48*, 605, Steinberg, 1931, and Robertson and his collaborators, 1939). For instance, Robertson and Fox, studying the outcome of experimental lobar pneumonia in the dog, report that the infecting dosage or the extent of pulmonary involvement, the leukocytic response, *and* the bacteremia [illegible] factors in prognosticating [illegible] ns to exist in various [illegible] ndered (cf Gay and [illegible] tamatis, *Ann. Inst. Pasteur*, 1939, [illegible]).

In brief, the sum total of all these observations clearly indicates the rôle of the inflammatory reaction in regulating the rapidity of invasiveness of a microorganism, and therefore the significance of this factor in grading the immunity or resistance of the host is manifest. However, the rate of dissemination in no way alters the virulence *per se* of the bacteria, which still remains a most important factor in evaluating resistance. Virulence and invasiveness are therefore regarded as two separate variables in studying problems of immunity (Menkin, 1935, 1936*c*).

The rapidity and intensity of local fixation in an inflamed area are factors to be reckoned with in determining the invasiveness of an infectious microorganism. Dissemination seems thus to be related directly to time and inversely to the degree of local injury. This may be conveniently expressed, perhaps, as follows:

$$D = \frac{Kt}{I}$$

where D refers to dissemination, t to time, I to extent of injury, and K is a constant depending on the physical properties of the irritant and perhaps on the anatomical position of the lesion (Menkin, 1938). Powerfully necrotizing irritants produce, as a result of an increase in capillary permeability and lymphatic damage, an extremely prompt "walling-off" reaction (termed *fixation*). By this early process, the area of injury is mechanically circumscribed and the dissemination of the irritant is prevented. This initial response, which may occur within thirty minutes following the injection of an irritant, allows a definite interval of time for the leukocytes to assemble at the site of inflammation for phagocytosis. *Staphylococcus aureus* is an example of such a bacterial irritant. Aleuronat is a chemical irritant of similar potency (Menkin, 1929). Mild irritants, on the other hand, produce only a delayed reaction thus allowing relatively free penetration of the irritant into the circulation for a considerable interval of time. Occlusion of the draining lymphatics in such

instances often takes place as late as two days following the introduction of the irritant. Hemolytic streptococci exemplify this type of irritant (Menkin, 1933). Another instance has been demonstrated by McMaster and Hudack who showed that, up to 48 hours following a mere skin incision or local burn, lymph drainage is adequate (1934). Subsequently lymphatics failed to convey effectively materials contained in them. The intensity of fixation is found frequently to parallel the extent of inflammatory edema. This, as already mentioned, would suggest that in an inflamed area the local swelling is at least in part the result of blockage to normal lymphatic drainage which is thus unable to cope adequately with the excess outpouring of plasma from the capillaries.

An interesting method for the study of bacterial infection and invasion has recently been devised by Goodpasture and his collaborators (1933, 1937). This consists in inoculating the chorio-allantoic membrane of chick embryos. The tissue behaves as a sterile living culture medium on which various types of bacteria may be inoculated. A means is afforded of propagating pure bacterial cultures in an organism and of thus studying the interaction of host and parasite. Various microorganisms were inoculated, including among others, cultures of *Staphylococcus aureus*, hemolytic streptococci, *C. diphtheriae*, *E. typhi*, and *Myco. tuberculosis avium*. With the exception of *Staph. aureus*, *Str. hemolyticus*, and *C. diphtheriae*, all other pathogens studied were found to multiply rapidly in the embryonic cells of the host. These microorganisms seem to be able to invade living tissues only through the agency of viable cells. Phagocytosis under these circumstances favors invasion and therefore does not act as a mechanism of defense. These investigators conclude that phagocytosis may thus have an ambiguous implication. It may under some circumstances favor extension of an infection while at other times it tends to eliminate bacteria. These instructive observations indicate the complexity of factors which may be involved in the problem of bacterial invasive-

ness. It is quite clear, however, from the studies of Goodpasture that in infections due to staphylococci or hemolytic streptococci their invasiveness in tissue is evidently not favored by phagocytosis, for these microbes are destroyed by leukocytes.

There is an additional significant factor that seems to play a rôle in the local dispersion of microorganisms. Duran-Reynals (1933) demonstrated that certain strains of staphylococci and streptococci contain a soluble factor capable of increasing tissue permeability. The filtrates of these organisms induce a spread of the local lesion. This property is apparently similar to the diffusion of India ink when injected along with testicular extract (Duran-Reynals, 1928, 1929). These observations recall the extensive pneumococcal type of lesion that Goodner described in the dermis of rabbits (1928). The latter also noticed the presence of an anticoagulating factor in the autolysates of pneumococcus cultures (1931). This property seems to favor the local spread of the edematous fluid containing pneumococci and allows the inoculation of all tissues which it reaches (Rhoads and Goodner, 1931). The writer, as described above, observed that the injection in the rabbit of broth cultures of Type I Pneumococcus seldom induces any appreciable fibrin deposits in the affected tissue areas. However, the ultimate formation of thrombi in lymphatics prevents the penetration of a vital dye to the tributary lymphatic nodes (Menkin, 1933). McClean noted the presence of a similar spreading factor in various members of the gas-gangrene group (1936). Pradhan, studying hemolytic streptococci (1937), likewise confirmed these findings. Some of these investigators conclude that the presence in various pathogens of the spreading factor induces an increase in tissue permeability which in turn seems to be of significance in determining the local invasiveness of a microorganism. They believe that the factor of invasiveness is independent of virulence. Their inference, as discussed above, is in agreement with the findings of Menkin (1936*c*). In reviewing the various studies on the spreading factor, Duran-Reynals

(1936) suggests the possibility that the development of lymphatic blockade may play a part in restricting the full effect of the spreading factor contained in some pathogens. The early "walling off" induced by staphylococci (Menkin, 1933) may thus counteract the effect of the spreading factor which they contain. On the contrary, the widespread types of streptococcal lesions are perhaps referable to the free play of the spreading factor since the development of lymphatic obstruction is considerably delayed. These studies and interpretations are doubtless of great interest. It is questionable, however, in the writer's mind whether the extent of local lesions is of major significance in evaluating invasiveness to the systemic circulation. In the last analysis this seems to be the main problem in regard to the organism as a whole. Although the extent of local invasiveness is doubtless of some significance, the mechanism of systemic invasiveness seems to be primarily associated with the capacity of microorganisms to reach ultimately the general circulation. As has been noted above, this seems to be a function of the local inflammatory lesion. The patency of the draining lymphatics, through which bacteria disseminate in order to reach the systemic circulation is determined primarily by the degree of local injury. *Inflammation thus becomes a basic factor in immunity as a regulator of generalized invasiveness.*

Summary. An important factor concerned with the mechanism of invasiveness by pathogenic microorganisms consists in the rapidity and intensity with which lymphatic blockade is established by their presence in otherwise normal tissue.

A culture of *Staphylococcus aureus* or of its soluble toxin induces prompt lymphatic occlusion, as indicated by histological examination of the site of inoculation, and also by the fact that within one hour after infection an injected dye, used as an indicator, fails to reach the regional lymphatic nodes. In the case of hemolytic streptococci, however, about two days are required after their cutaneous inoculation before the local reaction is of sufficient intensity to induce lymphatic

obstruction. These observations suggest a paradoxical interpretation. Staphylococci produce in general little systemic effect owing to their intense local injurious reaction which fixes them *in situ*. Streptococci produce marked generalized effects on the organism as a whole because of their comparatively mild local reaction. This allows them relatively free penetration to the essential organs.

Various other possible factors to account for the respective differences in the invasive behavior of the above pyogenic microorganisms are discussed. It is pointed out that neither the fibrinolytic factor contained in cultures of hemolytic streptococci, if at all present *in vivo*, nor the clotting principle or staphylocoagulase of *Staphylococcus aureus* can adequately explain the primary mechanism of invasiveness. The available evidence indicates that the capacity of pathogenic microorganisms to disseminate from their site of inoculation is apparently inversely related to the intensity of induced local injury. The patency of regional lymphatics determines the invasiveness of a microorganism.

Invasiveness and virulence constitute two separate variables in the grading of resistance. By introducing an irritant which induces prompt "walling off" of an area, dissemination of a virulent microorganism can be retarded and thus the full effect of its virulence on the host markedly delayed. Nevertheless, the virulence *per se* of the pathogen remains essentially unaffected by interfering with its dissemination. The observations described in this chapter indicate the important rôle of inflammation in immunity as a regulator of invasiveness.

CHAPTER XIV

RECAPITULATION AND CONCLUSIONS

In the foregoing chapters of this monograph observations have been recorded pertaining to the disturbed physiological and biochemical equilibria occurring in an area of acute injury. *The immunological implications of these disturbances* have led us to the formulation of a dynamic concept of the inflammatory reaction in relation to the organism as a whole. This viewpoint can now be briefly summarized somewhat as follows:

Inflammation may be regarded as the physical basis of infectious processes. The inflammatory reaction is initiated by a derangement in local fluid exchange. It subsequently proceeds through a series of interdependent sequences which ultimately *tend to localize and dispose of the irritant.* The disturbances in the local physiology of inflamed tissue are closely associated with the immunological significance of the inflammatory reaction. The principal sequences in the development of inflammation may be listed as follows:

a. *Increased fluid passage through the capillary endothelial wall.* This seems to be primarily referable to two factors: 1. *Elevation in capillary pressure* which is perhaps the outcome of a local axon reflex affecting the caliber of arterioles. 2. *Increased capillary permeability;* this seems to be referable to the liberation by injured tissue of the permeability factor termed *leukotaxine.* Leukotaxine is a nitrogenous substance, the significant properties of which evidently do not resemble those of histamine. It appears as if it may belong to the group of relatively simple polypeptides. It is postulated that its formation occurs as a result of the presence of an irritant interfering with local protein catabolism.

b. *Localization of the irritant* (fixation). The "walling off" of an inflamed area seems to be due to an enhanced passage of fibrinogen through the more permeable capillary wall. The mechanism of fixation is primarily referable to the formation of a fibrinous network and of thrombi occluding the lumina of draining lymphatics. This favors the development of lymphatic blockade in acute inflammation. Various secondary factors, such as the presence of immune bodies in anaphylactic or allergic inflammation, may reinforce the basic mechanism. The early occurrence of fixation in a severely injured area plays a definite rôle in immunity for it allows an interval in which the relatively sluggish leukocytes assemble for the purpose of phagocytosis.

c. *Migration of leukocytes.* The first cells to migrate into an inflamed area are the polymorphonuclear leukocytes. The mechanism of their migration seems to be related to the liberation of *leukotaxine* by injured tissue. The properties of the crystalline nitrogenous substance recovered from exudates offer a reasonable explanation for two of the basic sequences of the inflammatory reaction: first, the initial increase in capillary permeability, and secondly, the rapid emigration of polymorphonuclear leukocytes into injured tissue. Leukotaxine appears to contain factors concerned both with permeability and chemotaxis.

The polymorphonuclear cells are gradually displaced by macrophages. Cytological changes in acute inflammation seem to be conditioned by the pH of the exudate which in turn is often referable to disturbance in the local intermediary carbohydrate metabolism. The development of a local acidosis, resulting from increased glycolysis and depletion of the alkali reserve, seems to injure the polymorphonuclear cells. Macrophages survive and predominate when the pH falls to a level of about 6.9 or 6.8. Further reduction in the pH proves lethal to all types of leukocytes and frank suppuration ensues.

The rise in the level of circulating leukocytes, frequently encountered in infectious processes, seems to be referable

to the liberation in the inflamed area of a leukocytosis-promoting factor. This factor is either a globulin or at least it is associated with the pseudo-globulin fraction of exudates.

The interplay and dynamic relationships of the above sequences constitute an acute inflammation. An inflamed area can be considered as shunted off from the rest of the organism. It has its own metabolism, its own hydrogen ion concentration, and its own modified circulation. The inflammatory reaction thus displays an extraordinarily complex mechanism tending to localize and dispose of a chemical or bacterial irritant. This ultimately leads to organization and repair of the affected tissue.

The concept of fixation and its mechanism afford a rational interpretation of the rôle of inflammation in immunity. The capacity of pathogenic microorganisms to disseminate from their site of inoculation is apparently inversely related to the intensity of induced local injury. In this way inflammation plays a significant rôle in problems of immunity as a regulator of bacterial invasiveness.

A number of fundamental problems still remain unsolved. It is hoped that an attempt at their elucidation may loom as an attractive objective for the student of infectious processes. For instance, the exact nature of cellular injury both in regard to inflammation and to the development of neoplasia; the rôle of lymphocytes and plasma cells in certain inflammatory conditions; and the factors conditioning repair are only a few of the basic questions concerning which our information is as yet surprisingly meager. The introduction of various methods offered by the combined fundamental sciences of morphology, immunology, physiology, and biochemistry doubtless will throw further light on a problem which in all ages has been considered as the keystone to an understanding of pathology.

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AUTHOR INDEX